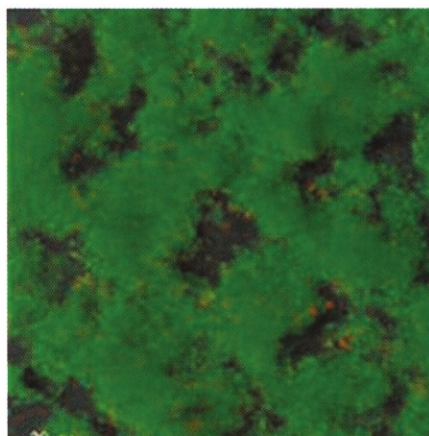
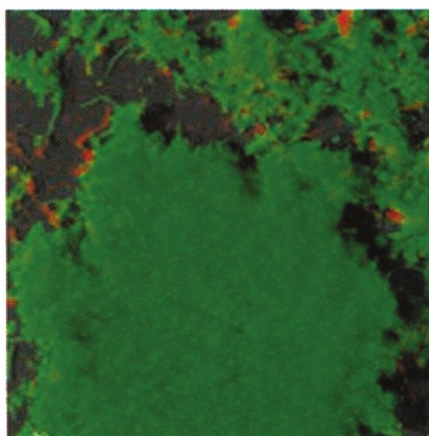

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Pseudomonas

*Genomics,
Life Style
and
Molecular
Architecture*



Edited by Juan-Luis Ramos

Pseudomonas

Pseudomonas

Edited by Juan-Luis Ramos, *CSIC, Granada, Spain*

Volume 1: Genomics, Life Style and Molecular Architecture

Volume 2: Virulence and Gene Regulation

Volume 3: Biosynthesis of Macromolecules and Molecular Metabolism

Pseudomonas

Volume 1
Genomics, Life Style and Molecular
Architecture

Edited by

Juan-Luis Ramos

CSIC

Granada, Spain

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PREFACE

The time was ripe for a series of books on *Pseudomonas* to see the light. About 18 years have passed since Jack Sokatch first published his outstanding *The Biology of Pseudomonas* back in 1986. This was followed by two books published by the ASM that contained the presentations of the *Pseudomonas* meetings held in Chicago in 1989 and Trieste in 1991. The earlier volume was edited by Simon Silver, Al Chakrabarty, Barbara Iglewski and Sam Kaplan, and the later by Enrica Galli, Simon Silver and Bernard Witholt.

The present series of books was conceived at a meeting with Kluwer staff members in August 2002 during the XI IUMS conference in Paris. In less than a year a group of outstanding scientists in the field, after devoting much of their valuable time, managed to complete their manuscripts for the three volumes of the series. It has been an honor for me to work with them.

The review process has also been of great importance to ensure the high standard of each chapter. Renowned scientists have participated in the review, correction and editing of the chapters. Their assistance is immensely appreciated. I would like to express my most sincere appreciation to:

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There has been growing interest in pseudomonads and a particular urge to understand the biology underlying the complex metabolism of these ubiquitous microbes. These bacteria are capable of colonizing a wide range of niches, including the soil, the plant rhizosphere and phyllosphere, and animal tissues; more recently they have attracted attention because of their capacity to form biofilms, a characteristic with potentially important medical and environmental implications.

There has been an explosion of vital new information about the genus *Pseudomonas*. A rapid search for articles containing the word “*Pseudomonas*” in the title in the last 10 years produces more than 6,000 articles! Consequently, we cannot cover all possible topics relevant to this genus in just three volumes, although our intention has been to be as thorough as possible. To organize the books, various topics have been grouped under a common heading, although this has some limitations since certain chapters may seem equally appropriate under different headings.

In Volume 1, the first chapter provides clues to the definition of the true *Pseudomonas* genus and gives a historical perspective of research to date. The insights in this chapter will undoubtedly help us to ascribe potential new isolates to this genus. Several recent advances in genomics, plasmids and phage biology, as well as a wealth of useful tools, are grouped under the heading “Genomics.” These chapters reveal the basis for diversity within the genus *Pseudomonas* at the molecular level, as well as key structural features. This section also describes a wide range of molecular tools to study gene expression and cloning in *Pseudomonas*, which have also been of great use in the analysis of other organisms. These chapters are therefore of potential interest to colleagues working in other genera in addition to *Pseudomonas*.

The section “Life styles” comprises a series of fascinating chapters that explain where pseudomonads can be found, their role in a particular niche, and how they interact with other members of the ecological community. Volume 1 concludes with a series of chapters on the architecture of *Pseudomonas* which explain the structure of the cell surfaces, their ability to

sense nutrients, and the use of the flagellar system of move toward chemoattractants or away from chemorepellent compounds. The molecular basis of cell architecture is critical to understand life styles, such as adhesion and secretion or extrusion of toxic substances including antibiotics and solvents. In all, I believe that the chapters included in this volume will by themselves constitute a book of general interest for microbiologists working on the biology of *Pseudomonas*.

Volume 2 deals with two important issues: virulence and gene regulation. The chapters under the heading "Virulence" deal with well-established virulence factors which make *Pseudomonas aeruginosa*, an opportunistic pathogen, and *Pseudomonas syringae*, a plant pathogen. Some issues on virulence were dealt with in Volume 1 under the heading "Life styles"; in Volume 2, the aim is to provide insights into the molecular mechanisms of virulence. In phytopathogens, their life style overlaps with some beneficial properties of non-pathogenic soil bacteria, but what actually makes them different is critical to our understanding of the biology of this group of *Pseudomonas*.

An astonishing finding from the analysis of the genomes sequenced to date is the wide battery of regulatory genes found in *Pseudomonas*. Also surprising is the wide range of alternative sigma factors encoded by these bacteria. Many of these factors belong to the ECF family and are involved in iron acquisition. The regulators of *Pseudomonas* can be placed into two broad groups, namely those that belong to the two-component class, in which a sensor protein "senses" the signal and triggers the actual regulator(s), and those of a second group in which sensing and regulatory functions are located in a single polypeptide. Volume 2 contains the most exhaustive and up-to-date compilation available of σ^{70} -dependent promoters in the genus *Pseudomonas*.

Volume 3 comprises the sections "Macromolecules," "Alternative respiratory substrates," "Catabolism and biotransformations" and "Secondary metabolism." The chapters in the section titled "Macromolecules" review some of the most complex metabolic pathways in the bacterial kingdom, and deal with the biosynthesis of LPS, fatty acids, alginate, rhamnolipids, cyclic lipopeptides, heme groups and vitamin B12.

Pseudomonads are well known for their extreme nutritional versatility and their ability to produce added-value products from simple, cheap carbon and nitrogen sources. The chapters in Volume 3 deal with the metabolism of certain amino acids and other natural compounds such as alkanes, as well as some xenobiotics and recalcitrant compounds such as aromatics. Some of the enzymatic properties of pseudomonads have been exploited to produce added-value products, which makes non-pathogenic *Pseudomonas* strains of great interest for certain industrial processes. This, in addition to the ability of certain strains to biosynthesize secondary products and influence the life style of certain strains, also makes them of interest for the industry.

In recent years, it has become clearer that pseudomonads are able to colonize anaerobic niches. In these niches, many strains are able to respire alternative electron acceptors, and the process of denitrification is indeed well understood in some strains of the genus *Pseudomonas*. More recently, a strain able to respire nitroorganic compounds has been reported, and recent studies have revealed that this property may be more widespread than was initially thought.

There remains no doubt in my mind that in the next 10 years we will see myriad articles dealing with *Pseudomonas* and shedding further light on our current understanding of the broad group of bacteria in the genus *Pseudomonas* as it is now defined. However, I am confident that this series of books has assembled a significant part of the current knowledge of *Pseudomonas* in the best possible manner. More importantly, I hope it will open new lines of research that will lead to a better understanding of this group of saprophytic microorganisms.

Last but not least, I would like to acknowledge the enthusiasm and assistance of Carmen Lorente in the compilation of the three volumes that constitute the *Pseudomonas* series.

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TAXONOMY

TAXONOMY OF PSEUDOMONADS: EXPERIMENTAL APPROACHES

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1. INTRODUCTION

Straight or slightly curved rods but not helical, 0.5–1.0 μm in diameter by 1.5–5.0 μm in length. **Most of the species do not accumulate granules of poly- β -hydroxybutyrate**, but accumulation of poly-hydroxyalkanoates of monomer lengths higher than C_4 may occur when growing on alkanes or gluconate. Do not produce prosthecae and are not surrounded by sheaths. No resting stages are known. Gram-negative. **Motile by one or several polar flagella**; rarely nonmotile. In some species lateral flagella of short wavelength may also be formed. **Aerobic, having a strictly respiratory type of metabolism with oxygen as the terminal electron acceptor; in some cases nitrate can be used as an alternate electron acceptor**, allowing growth to occur anaerobically. **Xanthomonadins are not produced**. Most, if not all, species fail to grow under acid conditions (pH 4.5 or lower). Most species do not require organic growth factors. Oxidase-positive or negative. Catalase-positive. Chemoorganotrophic. **Strains of the species include in their composition the hydroxylated fatty acids 3-OH 10:0 and 12:0, and 2-OH 12:0, and**

ubiquinone Q-9. Widely distributed in nature. Some species are pathogenic for humans, animals or plants. The mol% G+C content of the DNA is 58–69.

Type species: *Pseudomonas aeruginosa* (Schroeter 1872) Migula 1900, 884.

Such is the definition of *Pseudomonas* that appears in the new edition of *Bergey's Manual of Systematic Bacteriology*. It is a very formal definition, as it should be, and it does not convey the same emphasis expressed in the many papers describing the striking biotechnological capacity of these organisms, such as the degradation of a large number of organic compounds, the interactions with plants and associations in the rhizosphere that are advantageous for agriculture, or the dubious distinction of including one of the most dangerous, opportunistic pathogens among all bacteria. *Pseudomonas* and pseudomonads (i.e., *Pseudomonas*-like bacteria) comprise taxa of metabolically versatile organisms capable of utilising a wide range of organic and inorganic compounds and of living under diverse environmental conditions. Consequently, they are found ubiquitously in soil and water ecosystems and are also important as plant, animal and human pathogens^{138, 162}. Two important terms should be defined at this point to clarify an important nomenclatural distinction that may be obvious but is, apparently, often confusing. That is, '*Pseudomonas*' (capitalised and written in italics) is the validly published (i.e., with nomenclatural standing) name of a genus comprising bacterial species exhibiting the phenotypic characteristics described above. On the other hand, 'pseudomonad' (not capitalised and not italicised) is a descriptive term (i.e., '*Pseudomonas*-like'), with no formal nomenclatural status, accorded to a relatively nonexclusive collection of bacteria exhibiting some similarities to species of the genus *Pseudomonas*. Pseudomonad bacteria that may have been characterised according to only a limited selection of traits have been observed to be most similar to bacteria that previously had been identified (correctly or incorrectly) as *Pseudomonas* species. Unfortunately, orthodox taxonomic convention combined with a degree of uncertainty associated with the identifications of some pseudomonad bacteria has led, as well, to the point that the terms '*Pseudomonas*' and 'pseudomonad' are often used interchangeably and incorrectly. This observation may appear, at first consideration, to be pedantic and overly academic. However, an overriding tenet of taxonomy and nomenclature is the consistent communication of information about organisms. In order to communicate scientific information effectively, the terminology must be consistent. This is particularly true in the case of bacterial systematics, as more studies on microbial diversity are being reported and the biotechnological applications of microorganisms require uniform and standard protocols and methods of interpretation of data. The complexity of *Pseudomonas* and pseudomonads are shown in the following chapters, in which the spectrum of genetic, metabolic and ecological diversity of pseudomonad and *Pseudomonas* species are described in extensive detail.

2. HISTORICAL PERSPECTIVES AND CURRENT TAXONOMIC STATUS

The original creation of the genus *Pseudomonas* by Migula established a taxon based upon characteristics of cell morphology. It is perhaps important to use this opportunity to point out a discrepancy with respect to the publication date for the presentation of the genus. While the date for the publication of the genus *Pseudomonas* has been recognised as being 1894, Irwin Gunsalus⁷⁵ and Walter Zumft²³¹ have pointed out previously that the new genus was actually presented for the first time in 1895, by Professor Walter Migula at the Bacteriologischen Institut der Technischen Hochschule zu Karlsruhe in his compilation of a seven-year effort to collect and compare all described bacteria, 'Ueber ein neues System der Bakterien', published in the journal of his institute¹¹⁶. The initial description of *Pseudomonas* by Migula was based solely upon morphological characteristics, for example, type of flagella, as follows (translation):

Genus *Pseudomonas* nov. gen.

Cells with polar flagella. Endospore formation occurs in some species, but infrequently. (e.g., *Pseudomonas violacea*).

This inordinately succinct description established the new genus within the family *Bacteriaceae*, accommodating bacteria on the basis of observations of characteristic flagella type and the greenish or blue-green fluorescent pigment in the pus collected in bandages of hospital patients and wounded soldiers. A more comprehensive description of the new genus followed in 1900¹¹⁷ and registered the species *Pseudomonas aeruginosa* (previously described as '*Bacterium aeruginosum*'¹⁶¹ and ultimately to be the type species of the genus), as well as 75 other species, for which the detail of the phenotypic descriptions is remarkable, considering the time when the work was carried out.

Winslow *et al.*²¹⁶ established the family *Pseudomonadaceae*, encompassing the newly described genus *Pseudomonas* and a number of other disparate genera, many of which have been reclassified throughout the following years. However, many of the members of genera that had been categorised within the family *Pseudomonadaceae* have come to be regarded, at some point in time, as 'pseudomonads' and, by association, related to *Pseudomonas*. This has proven, on occasion, to be a source of confusion for microbiologists.

In more recent times, 29 well-characterised species of *Pseudomonas* were listed in the eighth edition of *Bergey's Manual of Determinative Bacteriology*, with another 206 less well-described species included as addenda⁵⁰.

This was the first compilation of the Bergey's series that included molecular data, that is, G+C content of genomic DNA¹⁰⁷, in the descriptions of the organisms, which was a significant addition to the descriptions included in the seventh edition of the Bergey's Manual. However, the level of taxonomic resolution between organisms that the %(G+C) of genomic DNA could afford was limited and, at that time, the data were handled simply as an additional determinative characteristic without any systematic weight.

The Approved Lists of Bacterial Names was published in the *International Journal of Systematic Bacteriology* in 1980¹⁶⁹, providing an inventory of bacterial species names described before 1980 to be formally recognised as having nomenclatural 'standing' under the requirements of the International Code of Nomenclature of Bacteria (1976 Revision). This list included 87 *Pseudomonas* species, effectively reducing the number of *Pseudomonas* nomenspecies by not including those names that were not consistent with the rules of the 'Code'.

A listing of 94 *Pseudomonas* nomenspecies was presented in the 1984 first edition of *Bergey's Manual of Systematic Bacteriology*¹³⁵. Many of the species were included with the acknowledgement of not having known relationships to the well-characterised species of the genus but with the recognition that conclusive analyses would eventually define their taxonomic positions. Within the *Bergey's Manual of Systematic Bacteriology*, perhaps the most significant contribution to the taxonomy of *Pseudomonas* was the presentation of the subdivision of the species of the genus on the basis of rRNA similarities, reflecting estimations of phylogenetic relatedness^{134, 144}. Five distinct 'rRNA homology' groups were observed to comprise: (a) 'group I'—*P. aeruginosa*, *P. fluorescens*, *P. putida* and related species, also termed the 'true' *Pseudomonas* (i.e., *Pseudomonas sensu stricto*), this group included species observed to cluster within Woese's γ -subdivision of the *Proteobacteria*^{176, 221}; (b) 'group II'—*P. cepacia*, *P. mallei* and related species (to be reclassified as *Burkholderia*), and *P. solanacearum*, *P. picketti* and related species (to be reclassified as *Burkholderia* and, subsequently, as *Ralstonia*), this group included species observed to cluster within Woese's β -subdivision of the *Proteobacteria*^{176, 220}; (c) 'group III'—*P. testosteroni* and related species (to be reclassified as *Comamonas*), *P. acidovorans* and related species (to be reclassified as *Comamonas* and, subsequently, as *Delftia*), *P. facilis* and related species (to be reclassified as *Acidovorax*), *P. palleronii* and related species (to be reclassified as *Hydrogenophaga*) and *P. saccharophila* (not reclassified, to date), this group included species also observed to cluster within Woese's β -subdivision of the *Proteobacteria*; 'group IV'—*P. diminuta* and *P. vesicularis* (to be reclassified as *Brevundimonas*), this group included species observed to cluster within Woese's α -subdivision of the *Proteobacteria*^{176, 219}; 'group V'—*P. maltophilia* (reclassified as *Xanthomonas* and subsequently as *Stenotrophomonas*), this

group included species observed to cluster also within Woese's γ -subdivision of the *Proteobacteria*. These data effectively established the basis for the framework for the modern taxonomy of *Pseudomonas*, as well as other pseudomonads. Since then, many of the organisms described originally as species of *Pseudomonas* have, in fact, been reclassified and new species have been described and their names validly published, largely due to the development and increasing application of methods for genotypic characterisation, which has been one, but not necessarily the only, means for assessing the 'natural' relationships of bacteria. The number of *Pseudomonas* species that are included in the second edition of *Bergey's Manual of Systematic Bacteriology* (in press at the time of this writing) amounts to 61, representing the number of species of the genus that have been described in the literature to the end of year 2000.

In the last few years, the changes in bacterial taxonomy have proceeded so rapidly that it is difficult to maintain an up-to-date overview of the taxonomic status of the individual species of any given genus. The status of the taxonomy of the genus *Pseudomonas*, including the reclassifications of species formerly included within this genus, and the addition of new species, can be observed on-line through the Internet at two principle sites: the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) site for 'Bacterial Nomenclature Up-to-Date' (<http://www.dsmz.de/bactnom/bactname.htm>); and the site of the École Nationale Vétérinaire, 'J.P. Euzéby—List of Bacterial Names with Standing in Nomenclature' (<http://www.bacterio.cict.fr>). These sites have compiled all the bacterial names of the 'Approved Lists of Bacterial Names'¹⁶⁹, as well as those that have been validly published since January 1, 1980. The sites are updated with the publication of each issue of the *International Journal of Systematic and Evolutionary Microbiology*, the sole journal and resource responsible for the valid publication of new bacterial names and nomenclatural combinations. Valid publication of a new species name within the genus *Pseudomonas*, or any bacterial genus, is made only by publication, as an original article or within the 'Validation Lists', in the *International Journal of Systematic and Evolutionary Microbiology*. Currently (June 2003), 148 species names of *Pseudomonas* are recognised with nomenclatural standing, although 48 of these species have been reclassified and are now considered to be basonyms or synonyms of species placed in other genera (see Table 1).

In this chapter, two aspects of the taxonomy of this important genus of Gram-negative bacteria are examined. The first has to do with the differentiation of *Pseudomonas* (*sensu stricto*) from other genera, including those with species that were collected under this name in the past, and the second presents the criteria used for the internal subdivision of *Pseudomonas* (*sensu stricto*) into species. For such analyses, the pertinent characteristics with taxonomic value must be selected from the complexity of the cells and used in the most effective way for genus and species differentiation. This may seem to be

Table 1. *Pseudomonas*^a species with validly published names and their current taxonomic status.

Species name	Type strain	Reclassification	Validation lists ^b and selected references ^c
<i>P. abietaniphila</i>	ATCC 700689		Nr. 70, 1999, [119]
<i>P. acidovorans</i>	DSM 39	<i>Delftia acidovorans</i>	[41], [144], [169], [186], [207]
<i>P. aeruginosa</i> ^d	ATCC 10145		[117], [144], [161], [169]
<i>P. agarici</i>	DSM 11810		[44], [169], [228]
<i>P. alcaligenes</i>	DSM 50342		[121], [144], [169]
<i>P. alcaliphila</i>	JCM 10630		[230]
<i>P. aminovorans</i>	DSM 7048	<i>Aminobacter aminovorans</i>	[41], [169], [193]
<i>P. amygdali</i>	DSM 7298		[44], [62], [149], [169]
<i>P. andropogonis</i>	DSM 9511	<i>Burkholderia andropogonis</i>	[44], [64], [169], [181]
<i>P. anguilliseptica</i>	DSM 12111		[169], [205]
<i>P. antimicrobica</i>	LMG 18920		[9], [35], [223]
<i>P. asplenii</i>	LMG 2137	<i>Burkholderia gladioli</i>	[8a], [44], [157], [169]
<i>P. aurantiaca</i>	ATCC 33663		[128], [169]
<i>P. aureofaciens</i>	LMG 1245	<i>P. chlororaphis</i>	[26], [93], [96a], [144], [169]
<i>P. avellanae</i>	DSM 11809		Nr. 61, 1997, [90]
<i>P. avenae</i>	DSM 7227	<i>Acidovorax avenae</i>	[44], [106], [169], [214]
<i>P. azotoformans</i>	JCM 2777		[84], [169]
<i>P. balearica</i>	DSM 6083		[19]
^e <i>P. beijerinckii</i>	DSM 7218		[45], [79], [169]
^e <i>P. beteli</i>	LMG 978		[44], [140], [157], [169], [201]
^e <i>P. boreopolis</i>	LMG 979		[45], [69], [169]
<i>P. brassicacearum</i>	DSM 13227	<i>Senotrophomonas maltophilia</i>	[1]
<i>P. brenneri</i>	DSM 15294		Nr. 87, 2002, [10]
<i>P. cannabina</i>	LMG 5096		[62]
^e <i>P. carboxydohydrogena</i>	DSM 1083		[114]
<i>P. caricapapayae</i>	LMG 2152		[44], [151], [169]
<i>P. caryophylli</i>	JCM 9310	<i>Burkholderia caryophylli</i>	Nr. 45, 1993, [24a], [144], [169], [182], [223]

<i>P. catleyae</i>	LMG 5286	<i>Acidovorax avenae</i>	[44], [146], [157], [169], [214]
<i>P. cedrina</i>	CIP 105541		Nr. 87, 2002, [36a]
<i>P. cepacia</i>	LMG 1222	<i>Burkholderia cepacia</i>	Nr. 45, 1993, [13], [143], [223]
<i>P. chloritidismutans</i>	DSM 13592		[222]
<i>P. chlororaphis</i>	DSM 50083		[144], [169], [179]
<i>P. cichorii</i>	DSM 50259		[144], [169], [181]
^s <i>P. cissicola</i>	LMG 2167		[24b], [169]
<i>P. citronellolis</i>	DSM 50332	<i>Burkholderia cocovenenans</i>	[165], [169]
<i>P. cocovenenans</i>	DSM 11318		[64], [169], [197]
<i>P. corrugata</i>	DSM 7228		Nr. 6, 1981, [158], [185]
<i>P. costantinii</i>	CFBP 5705		[125]
<i>P. cremoricolorata</i>	JCM 11246		Nr. 85, 2002, [192]
<i>P. delafieldii</i>	DSM 64	<i>Acidovorax delafieldii</i>	[144], [169], [215]
^s <i>P. denitrificans</i>	ATCC 19244		[49]
<i>P. diminuta</i>	DSM 7234	<i>Brevundimonas diminuta</i>	[144], [164], [169]
<i>P. doudoroffii</i>	DSM 7028	<i>Oceanimonas doudoroffii</i>	[16], [23], [45], [169]
<i>P. echinoides</i>	DSM 50409	<i>Sphingomonas echinoides</i>	[42], [144], [169]
^s <i>P. elongata</i>	DSM 6810		[45], [169]
<i>P. extremorientalis</i>	LMG 19695		[88]
<i>P. facilis</i>	DSM 649	<i>Acidovorax facilis</i>	[144], [169], [215]
^t <i>P. ficuserectae</i>	LMG 5694		[67]
<i>P. flava</i>	DSM 619	<i>Hydrogenophaga flava</i>	[169], [213]
<i>P. flavescens</i>	DSM 12071		[77]
^s <i>P. flectens</i>	LMG 2187		[44], [169]
<i>P. fluorescens</i>	DSM 50090		[117], [144], [169]
<i>P. frogi</i>	DSM 3456		[45], [169]
<i>P. frederiksborgensis</i>	DSM 13022		[6]
<i>P. fulva</i>	LMG 11722		[84], [169]
<i>P. fuscovaginae</i>	DSM 7231		[44], [118]
<i>P. gelidicola</i>	IAM 1127		[94], [169]
^s <i>P. geniculata</i>	LMG 2195		[169]

Table 1. Continued

Species name	Type strain	Reclassification	Validation lists ^b and selected references ^c
<i>P. gessardii</i>	CIP 105469		[203]
<i>P. gladioli</i>	DSM 4285	<i>Burkholderia gladioli</i>	Nr. 44, 1993, [169], [223]
<i>P. glathei</i>	DSM 50014	<i>Burkholderia glathei</i>	[169], [198]
<i>P. glumae</i>	DSM 9512	<i>Burkholderia glumae</i>	[169], [194]
<i>P. graminis</i>	DSM 11363		[18]
<i>P. grimonitii</i>	CIP 106645		[11]
<i>P. halophila</i>	DSM 3050		Nr. 29, 1989, [55]
^a <i>P. hibiscicola</i>	LMG 980		[44], [169], [201]
^a <i>P. huijensis</i>	DSM 10281		[103], [169]
<i>P. indica</i>	DSM 14015		[145]
<i>P. indigofera</i>	DSM 3303	<i>Vogesella indigofera</i>	[71], [117], [169]
<i>P. iners</i>	CIP 106746	<i>Martnobacterium georgiense</i>	[66], [84], [156], [169]
<i>P. jessenii</i>	CIP 105274		Nr. 70, 1999, [203]
<i>P. jinjuensis</i>	LMG 21316		[99]
<i>P. kilonensis</i>	DSM 13647		[168]
<i>P. korensis</i>	LMG 21318		[99]
<i>P. lanceolata</i>	ATCC 14669		[103], [169]
<i>P. lemoignei</i>	DSM 7445	<i>Paucimonas lemoignei</i>	[43], [91], [169]
<i>P. libanensis</i>	CIP 105460		[36b]
<i>P. lini</i>	ICMP 14138		[39]
<i>P. lundensis</i>	DSM 6252		[120]
<i>P. luteola</i>	DSM 6975		[8], [97]
<i>P. mallei</i>	ATCC 23344	<i>Burkholderia mallei</i>	Nr. 45, 1993, [144], [148], [169], [213]
<i>P. maltophilia</i>	DSM 50170	<i>Stenotrophomonas maltophilia</i>	[82], [140], [144]
<i>P. mandelii</i>	CIP 105273		Nr. 70, 1999, [203]
<i>P. marginalis</i>	DSM 13124		[44], [169], [184]
<i>P. marina</i>	DSM 4741	<i>Cobetia marina</i>	Nr. 88, 2002, [16], [169]

Table 1. Continued

Species name	Type strain	Reclassification	Validation lists ^b and selected references ^c
<i>P. putida</i>	DSM 291		[116], [144], [169]
<i>P. pyrrocinia</i>	DSM 10685	<i>Burkholderia pyrrocinia</i>	[169], [198]
<i>P. radiora</i>	DSM 1819	<i>Methylobacterium radiotolerans</i>	[70], [169]
<i>P. resinovorans</i>	LMG 2274		[45], [169]
<i>P. rhodesiae</i>	DSM 14020		Nr. 61, 1997, [34]
<i>P. rhodos</i>	DSM 2163	<i>Methylobacterium rhodinum</i>	[70], [169]
<i>P. rubrilineans</i>	LMG 2281	<i>Acidovorax avenae</i>	[169], [181], [214]
<i>P. rubrisubalbicans</i>	DSM 11543	<i>Herbaspirillum rubrisubalbicans</i>	[12], [169]
^c <i>P. saccharophila</i>	DSM 654		[144], [169]
<i>P. salomonii</i>	ICMP 14252		[60]
^c <i>P. savastanoi</i>	LMG 2209		[61], [144],
<i>P. solanacearum</i>	DSM 9544	<i>Ralstonia solanacearum</i>	Nr. 45, 1993, [144], [169], [223], [224]
<i>P. spinosa</i>	ATCC 14606		[102a], [169]
<i>P. stanieri</i>	DSM 7027		[17], [156]
<i>P. straminea</i>	JCM 2783	<i>Marinobacterium stanieri</i>	[84], [169], [191]
<i>P. stutzeri</i>	DSM 5190		[142], [144], [169]
<i>P. synxantha</i>	LMG 2190		[45], [169]
<i>P. syringae</i>	LMG 1247		[144], [169]
^c <i>P. syzygii</i>	DSM 7385		Nr. 34, 1990, [152]
<i>P. taeniospiralis</i>	DSM 2082	<i>Hydrogenophaga taeniospiralis</i>	[99a], [213]
<i>P. taetrolens</i>	LMG 2336		[45], [169]
<i>P. testosteroni</i>	DSM 50244	<i>Comamonas testosteroni</i>	[144], [169], [186]
<i>P. thermotolerans</i>	DSM 14292		[104]
<i>P. thivervalensis</i>	DSM 13194		[1]

<i>P. tolaasii</i>	LMG 2342	[44], [169]
<i>P. tremae</i>	CIP 106417	[62]
<i>P. umsongensis</i>	LMG 21317	[99]
<i>P. vancouverensis</i>	ATCC 700688	Nr. 70, 1999, [119]
<i>P. veronii</i>	DSM 11331	[53]
<i>P. vesicularis</i>	DSM 7226	[144], [164], [169]
<i>P. viridiflava</i>	DSM 11124	[20], [144], [169]
<i>P. woodsii</i>	LMG 2362	[44], [64], [169]

^aValidly published names of species of the genus *Pseudomonas*, invalid species names are not included.

^bValidation of the publication of new names and new combinations published outside the *International Journal of Systematic Bacteriology (IJSB)* or the *International Journal of Systematic and Evolutionary Microbiology (IJS&EM)*.

^cReferences of studies describing the new species, revised taxonomic status, or taxonomic/phylogenetic allocation.

^d*P. aeruginosa* is the type species of the genus.

^eBacteria that should not be included within the genus *Pseudomonas* but which have not been reclassified with a validly published name.

^f*Pseudomonas* species that should be reclassified with a new species name within the genus *Pseudomonas*.

ATCC = American Type Culture Collection, Manassas, Virginia, USA; CFBP = Collection Française des Bactéries Phytopathogènes, Station de Pathologie Végétale et Phytobactériologie, Beaucauzé Dedex, France; CIP = Collection de l'Institut Pasteur, Institut Pasteur, Paris, France; DSM = Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; IAM = Institute for Applied Microbiology, University of Tokyo, Tokyo, Japan; ICMP = International Collection of Microorganisms from Plants, Landcare Research, Auckland, New Zealand; LMG = Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium; JCM = Japan Collection of Microorganisms, The Institute of Physical and Chemical Research, Hirowawa, Wako-shi, Japan.

obvious and straightforward. However, species of the genus *Pseudomonas* and other pseudomonads are well known for their promiscuity in exchanging genetic material and the question of whether the traits of a given organism are stable and truly characteristic of such organisms becomes relevant taxonomically. Thus, the criteria for choosing the appropriate characteristics with 'taxonomic value', for the level of resolution being targeted, becomes the basis for any taxonomic analysis. In any case, the present chapter should be considered as a critical discussion of the methodological approaches for establishing a *Pseudomonas (sensu stricto)* and pseudomonad taxonomy, rather than a simple cataloguing of the organisms and their respective relationships.

3. DIFFERENTIATION OF *PSEUDOMONAS* FROM OTHER GENERA

Recognition of the phylogenetic heterogeneity of pseudomonads and the ability to differentiate *Pseudomonas (sensu stricto)* from phenotypically similar bacteria have been due, principally, to the development and application of methods for analysing bacteria at the molecular level^{2, 23, 43, 44, 45, 63, 93, 105, 144, 200, 211, 218}. De Vos *et al.*⁴⁵ proposed, and it is now generally accepted, that the genus *Pseudomonas* is limited to the species related to *P. aeruginosa* in the DNA-rRNA homology group I¹⁴⁴, within the γ -subclass of the *Proteobacteria*²²¹, now reorganised as the class '*Gammaproteobacteria*'⁹⁸. The species comprising this grouping sometimes have been referred to as the 'true' *Pseudomonas* or the 'fluorescent pseudomonads', due to the notable fluorescent, water-soluble pigments produced by *P. aeruginosa*, *P. fluorescens*, *P. putida* and some of the other well-known species of the genus, although, to add yet another note of discord to the enigma of pseudomonad and *Pseudomonas* taxonomy, not all species of the so-called 'fluorescent pseudomonads' actually produce fluorescent pigments, for example, *P. stutzeri*, *P. mendocina*, *P. alcaligenes*, to name but a few. In order to differentiate systematically *Pseudomonas (sensu stricto)* from other bacteria that years ago were included in the same genus or those that are newly isolated and characterised, the following characteristics may be considered.

3.1. Nucleotide Base Ratio of Genomic DNA

The first genotypic analysis applied in microbial taxonomy¹⁰¹, the nucleotide base ratio of genomic DNA, is measured as the ratio of the amount of guanine and cytosine nucleotides to the total amount of nucleotide bases (i.e., G+C content). Among prokaryotes, the G+C content may range between 22 and 74 mol%¹⁰⁸ and has proven to be useful for differentiating phenotypically similar taxa. Although no exact guidelines have been established,

generally, the difference in G+C content has been reported to not vary by more than 10 mol percent within a bacterial genus¹⁷⁵. Among the species of the genus *Pseudomonas* (*sensu stricto*), the G+C content will be seen to range from approximately 59 to 68 mol%, while the G+C content among species that have been reclassified within new genera may be observed to range from 65 to 69 mol% for species of the genus *Burkholderia*, 64 to 68 mol% for species of the genus *Ralstonia*, 62 to 70 mol% for species of the genus *Acidovorax*, 62 to 67 mol% for species of the genus *Comamonas*, 65 to 67 mol% for species of the genus *Brevundimonas*, to name a few examples¹³⁵. Thus, while markedly different genomic DNA G+C content certainly will reveal phylogenetically different bacterial taxa, similar G+C contents do not necessarily indicate relatedness.

Determination of the genomic DNA G+C content traditionally has been expected to be included in the descriptions of species and genera. However, as can be seen from the examples above, the ability to differentiate genera may be lost in overlapping values and the usefulness of the method is limited to those instances in which the strains also have been investigated for other properties.

3.2. Ribosomal RNA Gene Sequences

The rRNA sequence similarities between *Pseudomonas* species were determined for the first time by hybridisation of DNA to ribosomal RNA (rRNA) at the Department of Bacteriology at the University of California at Berkeley¹⁴⁴. The conservative nature of rRNA had been demonstrated in two laboratories^{48, 51} and was the source of inspiration for attempts to unravel the complex phenotypic heterogeneity observed among the various species of *Pseudomonas*. The experiments were performed using an adaptation of the DNA–DNA hybridisation technique, with total rRNA as one of the hybridisation partners⁹³. The original hybridisation methodology not only was less precise than rRNA sequencing would prove to be, but it required the isolation of substantial amounts of rRNA, which is not an easy task, due to its inherent instability. However, the careful use of the hybridisation methodology clearly revealed an internal subdivision of *Pseudomonas* into five distinct rRNA groups that corresponded to the levels of rRNA similarity observed between different genera or, perhaps, different families. The analyses were able, as well, to estimate the evolutionary relationships between the fluorescent pseudomonads and *Escherichia coli*, *Stenotrophomonas* [*Pseudomonas*] *maltophilia* and *Xanthomonas* species. The observed subdivision of *Pseudomonas* was confirmed in numerous experiments performed throughout the following years in many other laboratories^{26, 43, 44, 45, 132, 183}.

Subsequent to the analyses using the DNA–rRNA hybridisation methods described above, another approach targeting the ribosomal RNAs was being

developed and applied in the laboratory of Carl Woese at the University of Illinois. Their approach utilised a protocol of ribonuclease T or ribonuclease U₂ digestion of ribosomal RNA and sequence determination of the resulting oligonucleotides to produce characteristic 'catalogues' that were taxa specific⁵⁷. In a timely publication, 'What isn't a pseudomonad: the importance of nomenclature in bacterial classification'²¹⁸, Woese and his colleagues described the diversity of the pseudomonads along the same divisions that had been observed by the Berkeley group and effectively confirmed the pertinence of the name '*Pseudomonas*', that is, 'false unit'. Essentially, some species that were classified as members of the genus *Pseudomonas* were recognised to be less similar genotypically to other pseudomonads than they were to non-pseudomonad bacteria, for example, enterics, phototrophs, nitrogen-fixing plant symbionts, etc. With such approaches, the 'natural' system of bacterial classification that had been proposed to be theoretically feasible by microbiologists such as C.B. van Niel²⁰², but which had remained so elusive practically, was being acknowledged to offer significant possibilities for bacterial systematics

While the DNA-rRNA hybridisation and rRNA oligonucleotide cataloguing methods were able to provide overviews of the phylogenetic relationships of bacteria, the proponents of the methods acknowledged their respective limitations in being able to differentiate the closely related organisms. To this end, complete sequence determinations of the rRNAs were seen as the means for establishing a phylogeny-based bacterial systematics with the resolution to differentiate even the most closely related organisms^{100, 217}. Probably the most significant breakthrough, with respect to the determination of nucleic acid sequences for bacterial taxonomy, came with the development of the polymerase chain reaction (PCR)¹²⁴. PCR, combined with advances in DNA sequencing^{5, 83, 147}, enabled the determination and comparison of rRNA gene sequences (i.e., rDNA) to become a practical methodology in most microbiology laboratories, for rapidly analysing large numbers of organisms^{52, 111, 206}. The use of rRNA/rDNA sequence data for bacterial phylogeny and taxonomy had the added advantage of being 'additive'. That is, the rRNA/rDNA sequence of each newly characterised organism is compared to reference sequences in a database and then is added to the database to act, in turn, as reference for subsequent comparisons. Thus, the number of prokaryotic small subunit (i.e., 16S) rRNA/rDNA sequences compiled in databases has grown from less than 300 in 1990¹²⁷ to nearly 33,000 (nearly full-length) as of June 2003 (<http://rdp.cme.msu.edu>), available at the Ribosomal Database Project³². Of these, only five 16S rRNA sequences of *Pseudomonas* species were available in 1990. Currently, more than 1,300 16S rRNA/rDNA sequences of *Pseudomonas* species are available in the public databases.

The differentiation and inferred phylogenetic relationships of the various pseudomonad rRNA similarity groups, as well as of the species included in each one of the groups by comparative analysis of 16S rRNA gene sequences

has been published in two comprehensive articles^{7, 95}. Not surprisingly, the phylogenetic relationships inferred from 16S rDNA sequence analyses correlate with the earlier results determined from rRNA–DNA hybridisation and rRNA cataloguing data. Thus, species retained within the genus *Pseudomonas* (*sensu stricto*) are observed to be those closely related to *P. aeruginosa*, in the *Gammaproteobacteria*, while other species are seen to cluster within genera of the *Alphaproteobacteria* and the *Betaproteobacteria*. In fact, a substantial advantage that 16S rRNA/rDNA sequence comparisons have over the earlier methodologies for rRNA analyses is the possibility to observe directly the levels of similarity between *Pseudomonas* and closely related genera. For example, the bacteria most closely related to the genus *Pseudomonas* include the species of the aerobic, free-living *Azotobacter*–*Azomonas* complex, cellulolytic species of the genus *Cellvibrio*, marine bacteria of the genera *Microbulbifer* and *Marinobacterium*, endosymbiotic bacteria of the genus *Teredinibacter* and the halophilic bacteria of the genera *Halomonas*, *Oceanospirillum*, *Marinomonas*, and somewhat more distantly related to the *Moraxellaceae* family, *Marinobacter* species isolated from marine environments and methylo-trophs of the *Methylococcaceae*^{7, 95}. All of these organisms exhibit relatively diverse phenotypes and probably would not be suspected as being evolutionary ‘cousins’ of *Pseudomonas*, based upon traditional analyses.

The similarities in rRNA gene sequences and the phylogenetic relationships may be used, in turn, for developing tools for detecting and identifying bacteria at the genus and species levels in environmental or clinical samples. The high specificity of nucleic acid probes and the different regions of the rRNA molecule, containing more- and less-conserved sites, provide ready targets for oligonucleotides labelled with an appropriate molecule for *in vitro* or *in situ* detection of bacteria at different taxonomic levels³. Genus- and species-specific oligonucleotide probes have been designed^{4, 159} that enable the detection of combinations of pseudomonad species from the different proteobacterial classes. However, a set of hybridisation probes for differentiating all species of the various pseudomonad genera or all species of the genus *Pseudomonas* has yet to be developed. While it is possible to differentiate bacteria on the basis of a single base change in a probe target region, the sequence regions exhibiting the nucleotide differences (i.e., ‘signatures’) between species of a genus are limited, such that being able to resolve them with absolute confidence is not possible on the basis of a single nucleotide difference. Thus, Amann *et al.*³ have adopted the use of nested probes in an elegant ‘top-to-bottom’ approach, such that a combination of oligonucleotides, differentiating organisms at different taxonomic levels, is able to systematically refine the level of identification that can be determined. Such a strategy, which may require a large number of probes for identifying an organism of a specific sequence type, becomes realistic when used with the micro-array hybridisation

systems that are being developed. Whether a suite of micro-array 'chips' for the range of pseudomonad and *Pseudomonas* species will be developed obviously will depend upon the applications that may arise.

In the same way that rRNA/rDNA sequence data have provided the means for developing hybridisation probes as tools for differentiating pseudomonads and *Pseudomonas* species, the sequence data has also been used for devising PCR-diagnostic assays of high specificity. Widmer *et al.*²¹⁰ combined specifically targeted PCR-amplification assays with restriction fragment length polymorphism (RFLP) analyses to develop a rapid assay for detecting 16S rDNA sequence types that cluster within the phylogenetic spectrum of *Pseudomonas* species.

3.3. Cell Lipid Composition

The lipid compositions of cell walls have been recognised to reflect the 'natural relationships' of bacteria for more than 30 years⁸⁶ and fatty acid, polar lipid, lipoquinone and polyamine profiles are regarded as valuable phenotypic markers of taxonomic value^{150, 189}. Whole-cell fatty acid methyl ester (FAME) profile databases are now available as commercial products for the analysis of data, for example, the Microbial Identification System from Microbial ID, Inc. (Newark, Delaware), which are able to identify bacteria at the genus level, in most cases.

In several laboratories, fundamental studies on the compositions of the cellular lipids of pseudomonads and other taxa have been described^{33, 87, 123, 132, 212}. Whole-cell fatty acids of 16:0 and isomers of 16:1 and 18:1 appear to be typical of most *Proteobacteria* and are of little diagnostic value in differentiating *Pseudomonas (sensu stricto)* from other pseudomonads¹⁹⁶. In addition to 16:1 ω 9c, 16:0 and 18:1 ω 7c, *Pseudomonas* species have the 3-OH 10:0, 3-OH 12:0 and 12:0 as their cellular fatty acids, as well as the Q-9 ubiquinone. Most species also have the saturated 12:0 2-OH component and the absence of significant levels of 3-OH 14:0¹⁹⁶. A small number of species may also produce 14:0 fatty acid. Although little work has been done on the polar lipid compositions of pseudomonads, phosphatidyl glycerol, phosphatidyl ethanolamine and diphosphatidyl glycerol ('cardiolipin') have been observed as the major components, with a number of uncharacterised minor components also present (B. Tindall, personal communication). These comprise the typical cellular chemical compositions of species of the genus *Pseudomonas (sensu stricto)*. The differential value of the lipid components of various bacterial taxa is found in the overall combinations of the lipid species, not necessarily in the presence of a single lipid component. Furthermore, not only qualitative, but also quantitative differences in some of the lipid components (i.e., cellular fatty acids) also may be a valuable taxonomic marker for pseudomonads and *Pseudomonas* species.

3.4. Metabolism of Amino Acids

Different pathways leading to L-phenylalanine or L-tyrosine are conserved in bacterial taxa²⁷ and analysis of key enzymatic features in these pathways, such as DAPH synthase, prephenate dehydrogenase, arogenate dehydrogenase, prephenate dehydratase and arogenate dehydratase, have been used to differentiate members of rRNA homology groups²⁶. The multibranched pathway of aromatic amino acid synthesis offers, not only a degree of variation in biochemical details not exhibited in the pathway of aliphatic amino acid synthesis, but also a richer source of regulatory information. For these reasons, Jensen and his collaborators chose the interconnected pathways of aromatic amino acid synthesis as a model for the study of the phylogenetic relationships among the groups of aerobic pseudomonads^{27, 28, 209}. The results of this work, which had significant taxonomic implications, were in overall agreement with the subdivision of *Pseudomonas* into the five distinct rRNA similarity groups, and they went a step further in confirming the subdivision, that is, determined by DNA–DNA hybridisation, of one of the rRNA groups¹⁴⁴.

The enzymatic analyses are simpler to carry out than are the procedures for comparison of similarities among rRNA/rDNA by hybridisation or sequencing. A blind enzymological study²⁶ correctly assigned the test strains to their respective taxonomic groups and demonstrated that the determinative procedure is a good tool indeed.

There are several pathways for the degradation of arginine by different species of the genus *Pseudomonas*. In one of the pathways, known as the arginine dihydrolase or arginine deiminase pathway, arginine is converted to citrulline and this, in turn, into ornithine and ATP, which allows the cells to maintain their motility for an extended time under anaerobiosis. This was, in fact, the origin of the discovery of this pathway many years ago^{78, 166, 170}. The presence of arginine deiminase can be detected by measuring the disappearance of arginine or, more simply, an increase in the pH of the medium, due to ammonia liberation¹⁸⁸.

Pathways of arginine degradation in *Pseudomonas* are characterised by key reactions: Arginine oxidase in *P. putida*¹⁷⁷; arginine dehydrogenase in *P. aeruginosa*⁸⁹; and arginine succinyl transferase in fluorescent species and *P. mendocina*, as well as in species of other genera of aerobic pseudomonads^{177, 178}. The arginine decarboxylase/agmatine deiminase pathway appears to be characteristic of *Pseudomonas* and is the source of polyamines. Studies in this field may have taxonomic significance^{188, 190}. The arginine dihydrolase system may no longer be considered characteristic of *Pseudomonas*, since it can be detected also in species of other genera and, on the other hand, some *Pseudomonas* species, such as *P. stutzeri*, give a negative reaction. However, analysis of arginine dihydrolase, in combination with other tests, still can be of phylogenetic interest. The pathway

may be a primitive remnant from the anaerobic conditions existing during the early life of our planet, when amino acids may have been available as metabolic building blocks as well as sources of energy.

3.5. Cellular Proteins

An approach for assessing relationships among different bacterial genera includes the comparison of amino acid sequences of selected homologous proteins¹⁶³. However, as protein sequence determinations are not a routine procedure, methods for determining the antigenic divergence of heterologous proteins, using antisera raised from reference proteins, can be used for estimating amino acid sequence differences.

Glutamine synthetase, an important enzyme involved in nitrogen metabolism, has been the object of immunological, comparative studies of pseudomonads and the results were identical to those of the nucleic acid hybridisation experiments¹⁵. Other immunological studies of selected enzymes, such as muconolactone isomerase¹⁸⁰, aliphatic amidases³¹ and histidine ammonia-lyases¹⁵⁴, have provided information about the degree of similarity among homologous proteins of pseudomonads. These studies, which have been commented on elsewhere^{133, 137}, aside from representing further confirmation of the internal subdivisions of *Pseudomonas*, have useful determinative applications.

In an immunological study of the relatedness of histidine ammonia-lyases (histidases) from *Pseudomonas* species, the Anti-P antibody was prepared, which appeared to be a diagnostic reagent for fluorescent species of *Pseudomonas*, but not for the nonfluorescent species¹⁵⁴.

Another approach that exploits the immunological differentiation of characteristic cellular proteins has been described by Tesar *et al.*¹⁸⁷, in which whole-cell protein electrophoresis patterns reacting with antibodies of appropriate specificity in a Western Blot enable identifications over a wide phylogenetic range. Monoclonal antibodies generated against *Pseudomonas*-specific epitopes of outer membrane proteins were able to differentiate the species of *Pseudomonas sensu stricto* from species of closely related genera.

Such analyses appear capable of being applied for different taxonomic levels, such as the differentiation of the genus *Pseudomonas sensu stricto*, although the immunological approaches may have their greatest value for intra-generic identifications, due to their potential for extremely high specificities.

4. THE INTERNAL SUBDIVISION OF *PSEUDOMONAS*

The species is the basic unit of biology and, as well, has been applied in microbiology, albeit sometimes in a relatively haphazard fashion. As the

traditional definition for 'biological species' does not apply in the case of prokaryotes, the intrageneric units in microbiology have been accepted somewhat empirically and guidelines for determining what is necessary for describing a bacterial species are relatively recently evolved in any systematic manner. In a provident review, Rosselló-Mora and Amann¹⁵⁵ discussed the complexities regarding the concept of the prokaryotic species, and have proposed guidelines for categorising what they have termed the 'phylo-phenetic species', that is, 'a monophyletic and genomically coherent cluster of individual organisms that show a high degree of overall similarity with respect to many independent characteristics, and is diagnosable by a discriminative phenotypic property'.

In the case of the genus *Pseudomonas*, after progressing through one or more of the approaches described in the sections above, one is left with a group of species for which, unfortunately, the amount of information available is quite uneven. It is simply vast in the case of the type species of the genus *P. aeruginosa*, primarily because of its medical significance, since it has been considered to be the most important of all opportunistic pathogenic species. The amount of information on this species is so enormous that in medical circles '*Pseudomonas*' was taken for many years to be synonymous with *P. aeruginosa*. The extensive database of information on *P. aeruginosa* also derives from the fact that it is the species of *Pseudomonas* for which genetic data has been considerably easier to obtain than for the other species and, therefore, an overall picture of the genome structure for members of the genus has not been achieved until recently.

In the cases of *P. fluorescens* and *P. putida*, the catabolic properties have dominated other details of the biology, including the recently growing amount of information on their stimulating effects on plant growth. Unfortunately, these two species are represented in nature by many different biovars and the species names have come to be recognised as lacking precise systematic meaning. Perhaps, due to better knowledge of the properties useful in determinative schemes, the isolations of bacteria, such as *P. mendocina*, *P. stutzeri* and other species of *Pseudomonas*, are being reported with increasing frequency from clinical samples. Generally speaking, species of the genus *Pseudomonas* have a well-deserved reputation, on the one hand, based on the beneficial biotechnological properties, for example, those that are active in the degradation of xenobiotic pollutants. However, on the other hand, the genus also is infamous, due to the inclusion of species that are notorious as opportunistic pathogens of humans, as well as phytopathogens that significantly impact the agricultural and economic well-being of entire regions of the world.

While some species of the genus *Pseudomonas* (e.g., *P. aeruginosa*) are homogeneous taxonomic units that can be relatively easily identified, other species include complex subdivisions, such as the *P. fluorescens* and *P. putida* 'biovars', 'pathovars' in the cases of *P. syringae* and *P. marginalis*, or 'genomovars' in the case of *P. stutzeri*. Such internal organisation is a reflection of the heterogeneity of bacteria that have been studied extensively and whose

characteristics have not conformed completely to the understanding of the taxonomist. Additionally, discrepancies between the phenotypic and genotypic properties have further hindered attempts to resolve the discrepancies. However, given these complications, the 'species' is the biological unit that purports to represent a systematic description of basic properties presented by the organism.

Criteria that can be used for *Pseudomonas* species differentiation and identification can be listed as follows.

4.1. Genomic DNA–DNA Similarity

Genomic DNA–DNA reassociation similarities have become the accepted molecular standard by which bacteria are classified at the species level⁹². Interestingly, the exact levels of DNA–DNA similarity that may be used to 'define' strains of a species have been proposed to range from approximately 60% similarity with $T_{m(e)}$ values of 6–9°C⁹² to approximately 70% similarity with T_m values of 5°C²².

The original DNA–DNA experiments were performed in the laboratory of Professor McCarthy at the University of Washington¹⁰⁹ and, back in Berkeley, these experiments were continued and applied to the analysis of the pseudomonads to complement the existing phenotypic data. As mentioned previously, the results confirmed conclusions, based upon phenotypic analyses, pointing to a degree of heterogeneity and fundamental differences among the species assigned to *Pseudomonas*, as it was known at that time. Within the genus *Pseudomonas*, as is usually the case for other bacterial genera, as well, the definitive differentiation of species of a genus is more effectively performed using the DNA–DNA hybridisation methodology. Many experiments were performed, which supported the subdivision of the genus on the basis of the phenotypic properties, and one of the last studies focused on the fluorescent group of species^{93, 139}.

4.2. Ribosomal RNA Gene Sequences

Although the analyses of rRNA/rDNA sequence similarities of bacteria has been invaluable for elucidating their intergeneric relationships, the application of rRNA/rDNA sequence analyses to the determination of the intra-generic relationships of bacteria has proven to be less successful. Given the early optimism for applying a principle of 'one rRNA sequence type = one bacterial species', the reality observed in the last decade has proven to be sobering in light of the conservative nature of the ribosomal RNAs, which has proven to be limited in being able to define the fine bacterial taxonomic structure lower than the genus level. Stackebrandt and Goebel¹⁷⁴ carried out an analysis of the correlation between levels of genomic DNA–DNA similarity determined by hybridisation and 16S rRNA gene sequence similarities and

have described the levels of 16S rRNA gene sequence similarity (or dissimilarity) that can define when a bacterium does not belong to a given species. However, a definition of bacterial species based upon 16S rRNA gene sequence similarities still has not been established.

The problem for bacterial systematists is that the rRNA genes are now recognised to have evolved slower than most of the rest of the genomes of bacteria. Thus, a dilemma sometimes appears in which the results from rRNA/rDNA sequence analyses do not correlate with important phenotypic traits. During the last decade, there has been a concerted movement in microbial taxonomy to adopt phylogenetic relationships inferred from genotypic analyses, particularly those derived from rRNA/rDNA sequence analyses, as a taxonomic 'framework' to which the finer details are added by phenotypic characterisation. Without further comment or discussion concerning the reliability of phylogeny-based taxonomy 'frameworks' (such debate would require a chapter in itself), suffice it to say that 16S rRNA gene sequences have been able to demonstrate considerable internal evolutionary structure within the spectrum of species included within the genus *Pseudomonas* (*sensu stricto*)^{7, 8, 122}. Moore *et al.*¹²² were able to observe two distinct intrageneric divisions that were designated: (a) The '*P. aeruginosa* intrageneric cluster'; and (b) the '*P. fluorescens* intrageneric cluster', for want of better labels. Most species of *Pseudomonas* that have been isolated and characterised to date group within one of these two clusters. Furthermore, the species of the genus *Pseudomonas* were observed to be linked through distinct 'evolutionary lineages' within the primary dichotomy of the genus. Anzai and colleagues, in studies including larger numbers of species, observed the same overall topography of the two major clusters, as well as the organisation of species comprising the evolutionary lineages, with some exceptions^{7, 8}. Admittedly, the *Pseudomonas* intrageneric clusters and evolutionary lineages mentioned here comprise, more or less, a subjective organisation of inferred 'natural' branching orders. However, the overall topography of the two major intrageneric clusters, as well as the species comprising the evolutionary lineages, have been observed also in non-ribosomal RNA-based analyses, using FAME and phospholipid fatty acid profiling¹⁹⁶ and SDS-polyacrylamide gel electrophoresis (PAGE) analysis of whole-cell proteins¹⁹⁵. There is still some question as to whether *P. putida* and closely related species are included as an evolutionary lineage within the '*P. fluorescens* intrageneric cluster' or comprise a separate, distinct 'intrageneric cluster'. As more species of *Pseudomonas* are isolated and characterised by 16S rRNA gene sequence comparisons, the resolution between the sequences of the individual species decreases and the branching order becomes less reliable. Such observations lead to a significant problem in using rRNA gene sequences for species identifications. Overall, 16S rRNA gene sequence similarities between species range from approximately 93 to 99.9% (given some degree of error in sequencing results, 16S rRNA gene

sequences of different species that may be identical cannot be ruled out)¹²². Even though the complete 16S rRNA gene sequences are approximately 1,540 nucleotides long, less than 10% (148 nucleotide positions) of the gene of species of the genus *Pseudomonas* appears to be susceptible to nucleotide base change and most of the positions that have been observed to vary between species are located within limited areas of hypervariable regions. If it were conceivable to apply the principle of 'one rRNA sequence type = one species', an estimate of the number of possible *Pseudomonas* species would be something in the order of $(4)^{148}$ (i.e., 4 = the number of nucleotides that can exist at any given variable position; 148 = the number of variable nucleotide positions), a high number indeed. However, bacteria that are known to comprise different species have been observed to possess identical 16S rRNA gene sequences⁵⁸. With such an observation on record, one cannot assume that a unique 16S rRNA gene sequence will characterise only a single bacterial species. This premise, of course, has important consequences for *Pseudomonas* and pseudomonad taxonomy and the analyses of *Pseudomonas* and pseudomonads in studies of microbial ecology and diversity.

Other potential targets for resolving the species of the genus *Pseudomonas* are the 23S rRNA genes. In general, the limited number of sequences that have been determined have indicated that the larger (c. 2,500 nucleotides) bacterial 23S rRNA genes possess a higher degree of sequence variation than do the 16S rRNA genes¹³¹. Christensen *et al.*³⁰ analysed selected, variable target regions within the 23S rRNA genes of some species of *Pseudomonas* and the data failed to provide a basis for identifying closely related species. However, the number of analyses on the 23S rRNA genes of bacteria, including species of the genus *Pseudomonas*, has remained limited, such that a conclusive indication of their value for resolving the fine intra-generic relationships is not yet completely evident.

4.3. Alternative Gene Sequences

The use of functional gene sequences as molecular chronometers has met with varied success. In general, the rates of change are greater than those observed for rRNA genes and, thus, they have been proposed as alternatives for estimating close (e.g., intrageneric) phylogenetic relationships. Probably the most significant studies on these alternative, functional gene sequence targets have exploited the *gyrB* gene that encodes the β -subunit protein of DNA gyrase (topoisomerase type II)^{226, 227}. The base substitution rates of *gyrB* within species of the genus *Pseudomonas* were observed to be greater than those of the 16S rRNA genes of the same organisms. The differences in rates of base substitution were proposed to be due to the difference in the number of sites available for neutral base substitutions. Interestingly, while the branching

order of the most closely related bacteria, such as different strains of *P. putida*, was observed to be much more defined by *gyrB* gene sequence comparisons than could be achieved with 16S rDNA sequences²²⁷, the overall branching order of the range of different species of *Pseudomonas* was essentially the same.

Studies targeting other genes with purported evolutionary chronometer characteristics and applied to *Pseudomonas* include the *rpoD* gene for the σ^{70} factor of the RNA polymerase²²⁶ and the *narG* and *nosZ* genes for nitrate reductase and nitrous oxide reductase, respectively⁴⁰. However, the number of species and strains analysed in these studies has been too limited to be able to determine whether these genes provide reliable estimations of *Pseudomonas* phylogeny.

Yet another potential target site for resolving the intrageneric relationships of *Pseudomonas* is the non-coding, intergenic, internally transcribed spacer (ITS) region between the 16S and 23S rRNA genes. This DNA is excised during the process of transcribing the ribosomal RNA during the synthesis of ribosomes and, thus, is exposed to much less evolutionary conservation than the rRNA genes. Guasp *et al.*⁷⁴ were able to resolve the various genomovars of *P. stutzeri*, correlating with the differentiation determined by DNA–DNA hybridisation analyses. Although the ITS sequence data for other species of *Pseudomonas* is sparse, these studies, and others analysing other bacterial taxa, suggest that the sequence analysis of these DNA regions may offer an alternative to the complex DNA–DNA hybridisation analyses for identifying bacterial species.

4.4. Substrate Utilisation

Since the taxonomy of *Pseudomonas* has been reviewed extensively^{133, 135, 137, 141, 179}, this subject will be treated here in a very summary manner. The nutritional screening analyses that has made *Pseudomonas* renowned since the beginning of the twentieth century⁴¹ is applicable, as well, to other chemoorganotrophic microorganisms. For instance, after many years of study on strains of *Burkholderia* ('*Pseudomonas*') *cepacia* as phytopathogenic agents, their profile of nutrient usage was the only way of realising their remarkable physiological properties^{13, 133, 135, 137}.

The core of the taxonomic project on *Pseudomonas* at Berkeley was a nutritional screening of strains, in which the capacity for growth at the expense of each of a long list of carbon compounds was estimated, applying a modification of the original methodology¹⁷⁹, consisting primarily of using the method of replica plating, taken from the genetic methodology. Other variations of the auxanographic technique have been described¹⁶⁰.

It is interesting to remark here that in one case of results obtained by den Dooren de Jong using the original method⁴¹, one strain of *P. putida* utilised 77 of the 200 compounds tested. The compounds included 6 carbohydrates, 10 alcohols, 10 saturated fatty acids, 3 unsaturated fatty acids, 17 amino acids, 9 amides and 7 amines. In the Berkeley laboratory, a strain of *P. putida* grew on 80 of 150 compounds tested. Moreover, a strain of *Burkholderia* ('*Pseudomonas*') *cepacia* utilised one third¹³ of those compounds for growth. Interestingly, *B. cepacia* was, until then, considered to be only a mild plant pathogen of no great interest.

Our knowledge of the ability of using novel catabolic pathways and their regulatory mechanisms has been expanded by the discovery of the participation of these organisms in the degradation of xenobiotic compounds under aerobic conditions or conditions of denitrification. These compounds are found in many environments as a consequence of human activities and strain-specific gene islands have been found integrated into conserved tRNA^{gly}. This type of genetic island is derived from mobile elements that, upon integration, would endow others in a given habitat. This is one way of conceiving the remarkable capacity of these organisms.

The data collected from the large collection of strains at Berkeley were a very attractive resource for numerical taxonomy and two salient publications are noteworthy. In one¹⁷¹, remarkably, the authors were able to produce a three-dimensional representation, by principal coordinate analysis, resembling closely the conclusions that had been obtained based on nucleic acid properties. In the other publication¹²⁰, the data clearly demonstrated the convenience of separating one of the clusters as a new species, *Pseudomonas lundensis*. Interestingly, one of the groups being examined at the same time in our laboratory, corresponded to a very similar phenotype, supporting the same conclusions¹⁴.

4.5. The Metabolism of Aromatic Compounds

The work on the metabolism of aromatic compounds actually preceded the taxonomic enterprise and, in fact, was a powerful inducer of it. Immediately after their start, though, the taxonomic findings greatly stimulated the biochemical work. During the early stages of the taxonomy work at Berkeley, it was concluded that the β -ketoadipate pathway was characteristic of the fluorescent organisms, but also of organisms distant from them, such as *B. cepacia*, but that it was absent of *Comamonas* and related forms.

After the publication of the data on this subject it was discovered that the *cis,cis*- and *cis,trans*- isomers of muconic acid could be used by the acidovorans group of organisms, to the point that these compounds could be used for the isolation of these organisms by means of enrichment procedures. Although

these compounds can be used endocellularly by the fluorescent species, the cells are not permeable to them. In the *acidovorans/testosteroni* group, the muconic acid isomers induce the β -ketopathway, but this does not occur upon growth on aromatic substrates¹⁵³. By means of the use of antibodies against crystalline preparations of two enzymes of the β -ketoadipate pathway, muconate lactonising enzyme and muconolactone isomerase isolated from *P. putida* biovar A, heterologous reactions were observed with preparations from *P. putida* biovar B, *P. aeruginosa*, all biovars of *P. fluorescens*, and *P. stutzeri*, but not with preparations from *B. cepacia* or species of the *Comamonas* group¹⁸⁰.

Interestingly, aside from the DNA–DNA hybridisation experiments that were being performed at the same time, this work provided evidence of *P. stutzeri*'s relationship with the fluorescent group, which was to receive further confirmation in later experiments using rRNA. The results also emphasised the differences between the fluorescent pseudomonad *P. stutzeri* cluster and other species (*B. cepacia*, *C. testosteroni*) that later were to be allocated to different rRNA similarity groups.

4.6. Organisation of Pili and Flagella

With minor exceptions, the cells of *Pseudomonas* cells are motile by means of flagella, and the number of flagella is a convenient taxonomic characteristic within the genus. Thus, the majority of *P. aeruginosa* cells have only one flagellum, while most of the other species of fluorescent pseudomonads have more than one. *P. alcaligenes* and *P. pseudoalcaligenes* are also characterised by having one flagellum per cell. In a minority of cells, it is possible to observe exceptional *P. aeruginosa* cells with two or three flagella. The flagellar number of this species has been the subject of recent genetic research³⁸ and it is supposed to be controlled by FleN, a putative ATP/GTP binding protein, and the disruption of the gene *fleN* results in multiflagellation of otherwise monoflagellated strains, and also in chemotactic defects. The flagella are inserted at the poles of the cells, although some exceptions have been observed, in one instance, in *P. stutzeri*¹⁴² and, in another, in *P. aeruginosa* (unpublished observations). These lateral flagella are thought to participate in swarming of the population^{142, 167}. *P. stutzeri*, *P. mendocina*, *P. alcaligenes* and *P. pseudoalcaligenes* all have one flagellum per cell and they are all nonfluorescent species. Therefore, with some exceptions, the number of flagella seems to be a characteristic of taxonomic interest.

Fimbriae and flagella have been studied in *P. aeruginosa* mainly because they are involved in pathogenesis. That is, they are components of virulence factors. These two elements have been observed in the species *P. aeruginosa* and *P. alcaligenes*, which are related by several other characteristics, as was

noticed in the nutritional screening studies. On the other hand, no polar pili were observed in the strains of *P. putida*, *P. fluorescens* or *P. chlororaphis* that were analysed.

In contrast to the other components of the outer membrane, the composition of fimbriae, thus far, has not revealed interesting taxonomic implications. However, the structures of these cell appendages are poorly known for most species. The low G+C content of the pilin genes in *P. aeruginosa* suggests that they may have been transmitted by horizontal transfer from other organisms with lower G+C content in their genomic DNAs²⁴. A table of codon usage has been constructed showing that the choice of third base is in accordance with the high GC content composition (67.2 mol%). Pilin genes, as a consequence of their lower GC content, have a different pattern of codon usage²⁰⁸.

4.7. Outer Membrane Proteins

The methods for detecting similarities or differences on the basis of these components are expensive or complicated, such that the use of the monoclonal antibody MA1-6, which is specific for a single epitope in a protein of the outer membrane, has been recommended¹²⁶. The lipoprotein is H2, found in numerous clinical isolates of *P. aeruginosa* and also in the members of a substantial collection of environmental isolates of the same species. No major differences were found among clinical strains⁷⁶ and in many related species, but not in unrelated ones. It is absent in *E. coli* and also in some *P. aeruginosa* isolates.

P. aeruginosa is considerably more resistant to antibacterial compounds, among them the antibiotics, and this is attributed, in part, to the low permeability of its outer membrane. On the other hand, this species is more sensitive than other fluorescent pseudomonad species to the action of the chelator, ethylenediaminetetraacetate (EDTA)²¹¹. There is a high content of phosphate in the outer membrane of *P. aeruginosa* and the chelator may remove the divalent cations, mainly magnesium, to which the stability of the cell wall may be due.

4.8. Genome Structure and Organisation

Studies on cellular proteins have been developed by making use of electrophoretic techniques and there is an abundant bibliography that is informative on this point^{36, 96, 195}. By far, however, comparative studies on the genome have received comparatively more attention from the taxonomic standpoint. A convenient technique makes use of enzymes that cut the genome infrequently, giving large genomic fragments that can be resolved by the use of pulsed-field electrophoresis. In some instances, enzymes cutting the chromosome in many fragments (e.g., *Hind* III) have been used in combination with multivariate analysis of the fragment patterns obtained on polyacrylamide gels¹⁷². However,

a limited number of cuts per genome has proven to be optimal for genomic restriction mapping for taxonomic use¹¹⁰, with clear advantages over the low resolving power derived from frequently cutting endonucleases^{46, 72, 73, 115}.

Application of this principle resulted in the proposal of a method for constructing and analysing macrorestriction patterns of 235 strains representing 32 species of *Pseudomonas*⁷³. Unsheared chromosomal DNA is prepared from cells included into low melting point agarose blocks, which, after treatment with lytic agents, are exposed to restriction endonucleases. Among these, *AsnI*, *DraI*, *SpeI*, *XbaI*, and *PacI*, appeared to be the most appropriate, since they are specific for AT-rich regions, or for sites that include the extremely rare tetranucleotide CTAG¹¹⁰. After pulsed-field electrophoresis, the macrorestriction patterns are compared by direct visual inspection (when they are very similar or identical), or, more appropriately, they are quantitatively evaluated by application of specially designed algorithms. After dividing the range of fragment sizes into a number of intervals, a similarity coefficient (Dice's coefficient, or S_D) can be estimated for each pair of strains by the number of intervals occupied by fragments of both strains. Use of appropriate equations often indicates a correlation between the estimated similarities of macrorestriction fingerprints, and conventional taxonomic groupings.

Strains sharing the same GC contents in the DNA, the same codon usage, and similar genome size, give coefficients placed above the confidence interval, and should be considered as members of the same taxon. The results obtained for strains of *Pseudomonas* species⁷³, in general, have confirmed the classifications based on an extensive amount of phenotypic and genotypic analyses, with some exceptions whose significance is difficult to evaluate at present. Obviously, differences in restriction fragment size distribution may be affected by chromosomal rearrangements and/or mutations at the restriction sites, in which case the restriction fingerprints should be supplemented with other analyses to determine the degree of similarity of the fragment patterns.

A less extensive study on pathovars of fluorescent plant pathogenic bacteria⁷² has indicated that two strains belonging to the same pathovar, but of different origins, can have almost identical restriction fingerprints. However, this is not always the case and it appears that wide host range pathogens may show more variation in their genomic fingerprint patterns than pathogens of a restricted host range.

4.9. Genetics of Alginate Production

Sequences homologous to four *P. aeruginosa* alginate genes have been detected in the chromosomal DNA of Group I *Pseudomonas* species, with the exception of *P. stutzeri*, which reacted to only three of four probes^{56, 68}. From the screening experiments, it was concluded that the alginate genes are found

in organisms of the genus *Pseudomonas*, *Azotobacter*–*Azomonas* lineage, and that fewer genes are present in species of the rRNA group V and the enterics.

In a related study, the trans activation of gene *algT*, which controls the conversion of *P. aeruginosa* to the mucoid phenotype, was found to be caused by a mutation in a gene *algN* of an adjacent locus, whose product presumably acts negatively in the regulation of alginate production⁶⁵. On the basis of an earlier report on the isolation of alginate-producing mutants of three species of *Pseudomonas* (*P. fluorescens*, *P. putida* and *P. mendocina*) by selection for carbenicillin resistance, a search was made for the presence of DNA fragments hybridising to an *algT* probe from *P. aeruginosa* in other *Pseudomonas* species⁵¹. Homologous sequences were detected in *EcoRI* digests of *Pseudomonas* species (*P. fluorescens*, *P. putida*, *P. mendocina* and *P. stutzeri*), but not in the DNA of *Comamonas* [*Pseudomonas*] *acidovorans* (rRNA group III), *Brevundimonas* [*Pseudomonas*] *diminuta* (rRNA group IV), or *Stenotrophomonas* [*Pseudomonas/Xanthomonas*] *maltophilia* (rRNA group V).

4.10. Siderophore Production

Siderophores are proteins excreted by most microorganisms to obtain their iron requirements. Siderophores effectively complex relatively insoluble iron forms in order to be internalised by specific membrane receptors¹¹². Pyoverdine is the major siderophore type of species of the genus *Pseudomonas*¹¹³ and the taxonomic value of siderophore production has been examined extensively. The approach has two aspects. On the one hand, the type of siderophore is determined (siderotyping), and this is supplemented by a study of the competition among the Fe-siderophores of diverse origins. The results from a large number of fluorescent and nonfluorescent strains correlated with the results of genotypic analyses, indicating that this approach is extremely promising for differentiation at the species level^{59, 112}.

5. SUMMARY

This review has attempted to present the current state of pseudomonad and *Pseudomonas* taxonomy in the light of a non-exhaustive description of methodologies that have been used for such analyses as the relatively recent development and recognition of new approaches for systematically defining bacteria. Unapologetically, the description of the new systematics of pseudomonads and *Pseudomonas* has been presented here with an emphasis on the genotypic analyses that have served to provide the data for establishing a taxonomic framework based upon estimations of phylogenetic relationships. Certainly, these data have comprised the majority of new taxonomic information

since the last two major compilations on pseudomonads and *Pseudomonas*^{80, 136}. However, it should be appreciated that each of the spectrum of methodologies described above, applied in polyphasic approaches, has played an important role in recognising the heterogeneity of *Pseudomonas sensu lato*, and in improving and stabilising the taxonomy of *Pseudomonas sensu stricto* and other pseudomonads. Hans-Jürgen Busse and colleagues²⁵ and Peter Vandamme and colleagues¹⁹⁹ have presented pertinent reviews on the importance of polyphasic approaches for bacterial taxonomy. Furthermore, even with recognition of the contributions that the genotypic methods have afforded, many questions regarding the intrageneric organisations of *Pseudomonas* and the various genera of pseudomonads still exist. It will be due to the systematic combination of analytical methods that will eventually unravel the taxonomic complexity of the various biovars of *P. fluorescens* and *P. putida*, the genomovars of *P. stutzeri* and the pathovars of *P. syringae*. Even though many of the methods that have been described here have indicated greater or lesser value for resolving bacteria at different taxonomic levels, some of them have not been tested extensively. As newer species of *Pseudomonas*, as well as pseudomonad bacteria, are isolated and described, the correlation of metabolic and genetic characteristics with the taxonomy of the organisms may shed light on their ecological and biotechnological potential.

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GENOMICS

THE GENOME OF *PSEUDOMONAS* *AERUGINOSA*

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1. INTRODUCTION

One of the remarkable properties of the common soil organism *Pseudomonas aeruginosa* is its ability to cause infections in a wide variety of hosts, including insects, plants, and animals²⁷. In humans, this organism can colonize virtually any mucosal surface and can invade tissues and blood. The infections can be of short duration and superficial, life threatening acute bacteremia, or chronic, spanning many decades, as in patients with cystic fibrosis^{25, 26, 36}. This ability to thrive in what may be arguably the broadest distribution of ecological niches for any bacterial species was expected to be matched by a complex, highly regulated genomic repertoire. Not surprisingly, early estimates of the genomic size suggested a relatively large genome exceeding 6 Mb⁴⁷. The completion of the sequencing project of the *P. aeruginosa* PAO1 genome in 2000⁵⁰ and the results from the second *P. aeruginosa* genome sequencing of the strain PA14 clearly demonstrate that the ecological diversity is indeed reflected in the gene content, with the coding capacity of the *P. aeruginosa* genomes comparable to that of a simple eukaryote *Saccharomyces cerevisiae*, at c. 6,000 open reading frames (ORFs).

2. *P. AERUGINOSA* GENOME SEQUENCING PROJECT AND PSEUDOCAP

The sequencing of the *P. aeruginosa* PAO1 genome reflects a highly successful collaboration between a group of academic investigators at the University of Washington and, a biotechnology company, PathoGenesis (now part of Chiron Corp.). The project was facilitated by the U.S. Cystic Fibrosis Foundation, which shared the cost of sequencing with PathoGenesis. Strain PAO1 was chosen for sequencing through an agreement among *P. aeruginosa* investigators, based primarily on the extensive use of this strain for genetic and physiologic studies in laboratories worldwide during the past five decades.

The project utilized what is now a standard two-phase strategy for sequencing of small to medium size genomes: a random “shotgun” sequencing approach followed by closure of contigs involving sequencing of specific targeted regions. Several M13 libraries were prepared from total DNA of *P. aeruginosa* PAO1 that were used to generate approximately 95,000 sequencing reads resulting in a 6.9-fold coverage of the genome. The finishing part of the project involved additional sequencing to generate high quality reads in the regions of low coverage from shotgun sequencing and closure of several contigs that terminated at repetitive sequences. The finishing phase required an additional 1,600 reads, using custom priming and extension of reads from the existing templates. Finally, a cosmid library of PAO1 DNA was made and reads from both ends of 800 randomly selected clones were generated. This sequence was then annotated using standard computational tools for the identification and assignment of genes (programs used were GeneMark.hmm²⁴ and BLAST¹). In addition to the evaluation of the gene’s codon usage, other considerations were utilized, primarily for the assignment of the start codon; these included the presence of a ribosomal binding site and when applicable, a predicted signal peptide (see Table 1).

One of the invaluable assets of the project became the participation of the *P. aeruginosa* research community in the genome annotation. The Pseudomonas Community Annotation Project, termed PseudoCAP, was established to provide an Internet-based platform for volunteers from the research community to submit annotations of genes with which they were familiar (usually because these genes were the subject of their individual research projects). The PseudoCAP organizers reviewed the submissions, and approved annotations were incorporated into the final annotation of the genome. Given the huge response and the breadth of the experts in the field who participated in the effort, the annotation of the *P. aeruginosa* PAO1 genome can be considered one of the most thorough and accurate of any bacterial genome sequencing project. The PseudoCAP concept has been retained on the current site

Table 1. A summary of some of the general features known about the *P. aeruginosa* PAO1 genome^a.

Size	6,264,403 bp
Terminus of replication	2,439,100 ± 5,000 bp
Overall %(G+C)	66.56
Regions of atypical %(G+C) greater than 3 kb	10
No. of copies of integrated bacteriophage genomes	2
Coding density	89.35%
RNA elements (% of genome)	0.39%
No. of ribosomal RNA (16S, 23S, 5S) genes	4 each
No. of transfer RNA genes	63
Open reading frames	
Protein coding sequences	5,570
No. of functionally classified genes	3,058
No. of conserved genes without a function	748
% AUG initiation codons	88.7
% GUG initiation codons	9.9
% UUG initiation codons	1.4

^aData sources were Kiewitz *et al.* ⁽¹⁵⁾ for the terminus of replication and the other characteristics were calculated using the updated annotation table (released on October 8, 2003) including data submitted by members of PseudoCAP and was taken from the Pseudomonas Genome Project web page (<http://www.pseudomonas.com>).

<http://www.pseudomonas.com/>, which allows the community to continue the annotation of not only unknown genes but correct any mistakes or omissions of the current annotation. There is little doubt that the PseudoCAP project contributed in a significant way toward the general utility of this particular genome-sequencing project to microbiological research.

3. REPRESENTATION OF FUNCTIONAL CATEGORIES AND COMPARISON TO OTHER MEMBERS OF THE *PSEUDOMONAS* GENUS

Functional assignments of products of predicted ORFs were carried out using relatively strict criteria. These were based on an identity or similarity of any one ORF to products of characterized genes of *P. aeruginosa* or other bacteria and minimally, the presence of a conserved sequence motif defining or associated with a putative biological function. In spite of the high stringency applied to the annotation phase of the genome project and the large number of genes in the genome, over 50% of the ORFs were assigned a functional class (see Table 2).

Expectations, based on the number of potential environmental niches that this organism can occupy, were met in the analysis of major functional

Table 2. Genes in the *P. aeruginosa* PAO1 genome summarized according to primary function^a.

Primary function	Number of genes	Percentage
Adaptation and protection	73	1.31
Amino acid biosynthesis and metabolism	154	2.76
Antibiotic resistance and susceptibility	19	0.34
Biosynthesis of cofactors, prosthetic groups, and carriers	132	2.37
Carbon compound catabolism	135	2.42
Cell division	26	0.47
Cell wall, LPS, and capsule	86	1.54
Central intermediary metabolism	65	1.17
Chaperones and heat shock proteins	52	0.93
Chemotaxis	40	0.72
DNA replication, recombination, modification, and repair	81	1.45
Energy metabolism	170	3.05
Fatty Acid and phospholipid metabolism	57	1.02
Hypothetical, unclassified, and unknown	2,370	42.55
Membrane proteins	43	0.77
Motility and attachment	77	1.38
Non-coding RNA genes	77	N/A
Nucleotide biosynthesis and metabolism	61	1.10
Protein secretion and export apparatus	84	1.51
Putative enzymes	447	8.03
Quinolone signal response	6	0.11
Related to phage, transposon, or plasmid	62	1.11
Secreted factors (toxins, enzymes, alginate)	61	1.10
Transcription, RNA processing, and degradation	45	0.81
Transcriptional regulators	402	7.22
Translation, post-translational modification, and degradation	149	2.69
Transport of small molecules	558	10.02
Two-component regulatory systems	115	2.06

^aThe number of genes in each primary function category was calculated using the updated annotation table (released on October 8, 2003), including data submitted by members of PseudoCAP, and was taken from the *Pseudomonas* Genome Project web page (<http://www.pseudomonas.com>). Percentage refers to 5,570 as the total number of genes, which excludes RNA genes.

categories. These include a large number (517) of genes encoding proteins with motifs of transcriptional regulators or members of two-component regulatory systems, corresponding to 9.3% of the genome. Another highly represented family of approximately 560 proteins are those that have been annotated as being involved in the transport of small molecules. Included in this category are transporters involved in import of nutrients, whose abundance further reflects the ability of *P. aeruginosa* to maximize the utilization of nutrients present in its potentially diverse environments. Further examination of

the genome of *P. aeruginosa* revealed in more depth the basis of its virulence involving a broad range of hosts. In addition to well-characterized toxins, the genome revealed a surprising redundancy in transport mechanisms which are dedicated to extracellular targeting of toxins and also a variety of adherence mechanisms. Transport mechanisms include several type II secretion systems with nearly all genes necessary to form the membrane associated secretion complex. Prior to completion of the PAO1 genome sequence, the type II secretion system represented by the 12-member *xcp* gene cluster, was believed to be responsible for the transport of the majority proteins secreted to the extracellular compartment. No fewer than five fimbrial adhesin gene clusters have been identified, although prior to genome sequencing, only type IV pili were known to mediate adhesion of *P. aeruginosa* to eukaryotic cells. Finally, the ability of *P. aeruginosa* to resist the action of a broad range of antibiotics had been partially attributed to the activity of one of the four efflux systems belonging to the resistance-nodulation-cell division (RND) family. However, it is apparent from the genome analysis that this organism has a potential to express a significantly larger number and variety of efflux systems including seven additional members of the RND family. This cursory examination of the functional genomic repertoire highlights the diversification of *P. aeruginosa* to environmental niches that require an unusually large number of what may appear to be redundant survival mechanisms. These include production of factors that promote colonization and adhesion of environmental or host tissue sites, utilization of available local nutrients, and the ability to overcome host defense mechanisms or the lethal action of antibiotics elicited by other organisms in the environment or when administered during treatment of infection. These transport and adhesion systems are discussed in detail in various chapters in this volume.

The availability of the genome sequence of two of the most closely related members of the genus *Pseudomonas*, *P. putida* KT2440³² and *P. syringae* pv. *tomato* DC3300⁵ provides an opportunity to compare the functional conservation of genes in all three organisms. Although these three *Pseudomonas* species are free living environmental bacteria, they do not share an identical lifestyle, particularly when it comes to interaction with other organisms. Although *P. putida* can often be isolated from decomposing organic material, it is considered to be avirulent, while *P. syringae* is a classical plant pathogen that does not have an ability to cause disease in animals.

A comparative analysis of the *P. aeruginosa* PAO1 genome with that of *P. putida* and *P. syringae* revealed that these organisms are indeed closely related. Using a BLAST cutoff of $E < 10^{-4}$, homologues of almost 4,500 (over 75%) *P. aeruginosa* genes can be found in *P. putida* and homologues of approximately 4,400 genes are found in *P. syringae*.

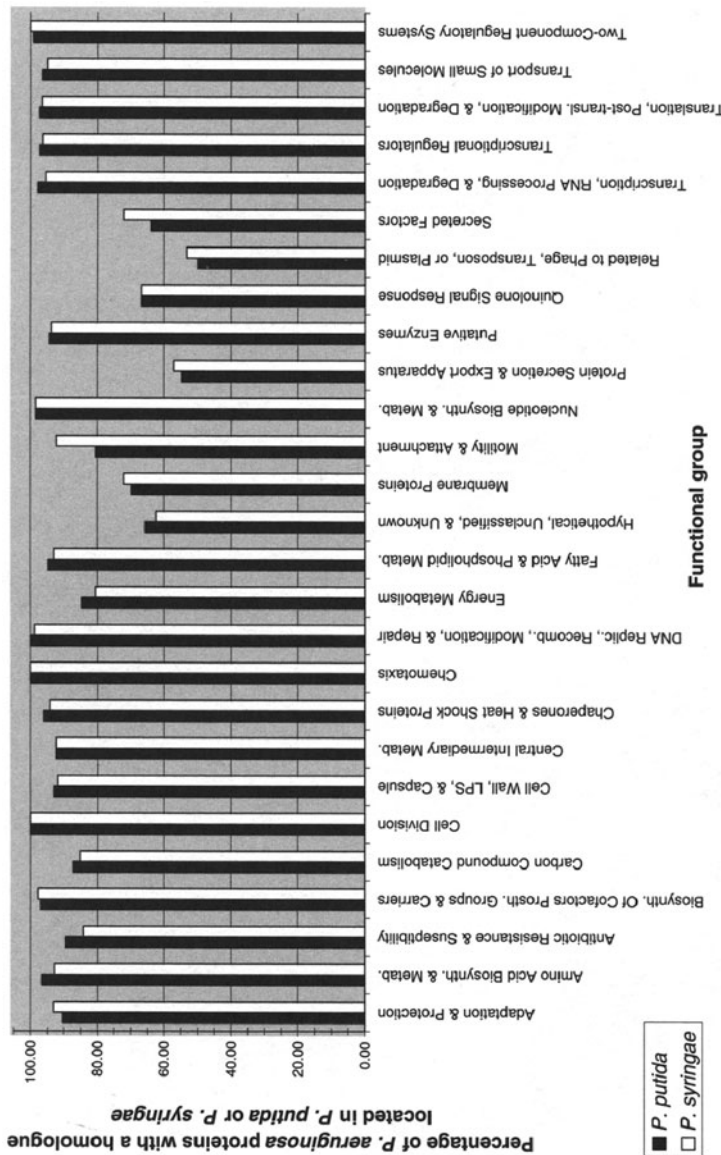


Figure 1. A comparison of the genome content of *P. aeruginosa* with that of *P. putida* and *P. syringae*. All protein sequences from 5,569 *P. aeruginosa* ORFs (excluding one with a frameshift mutation) were compared to the proteomes of *P. putida* and *P. syringae* using the BLAST algorithm (1) combined with the BLOSUM 62 matrix. Proteins that produced alignments were reported as homologues if the BLAST E value was less than 10^{-4} . The results were tabulated according to the functional groups of *P. aeruginosa* (listed in Table 1). Abbreviations are: biosynth., biosynthesis; metab., metabolism; prost., prosthetic; replic., replication; recomb., recombination; and transl., translation. Some groups contain highly conserved structures (such as two-component regulatory systems) and it is most likely that within these groups paralogues are being detected as opposed to orthologues.

A more informative overview of similarity is a comparison of annotated genomes in terms of functional categories. Figure 1 shows the representation of *P. aeruginosa* homologues in *P. putida* and *P. aeruginosa*, organized by functional classes. In general the distribution of genes shows a significant level of conservation across all functional categories. Notable functional categories that are underrepresented are mobile genetic elements (phages and transposons), which may be an indication of their limited host range to particular species. The underrepresented homologues belonging to the category of membrane proteins (mostly uncharacterized) are also significant. *P. aeruginosa* is thought to have one of the most adaptive capabilities allowing it to survive in diverse niches and the number of unique membrane proteins could reflect necessary mechanisms for survival in various environments. The functional category of small molecule transporters is not underrepresented, but is notable because in each member of the *Pseudomonas* genus, this category consists of components of nutrient transport systems whose specificities are closely related to the availability of nutrients in most environments that each organism inhabits. A significantly larger number of transporters specific for plant-derived sugars in the genome of the plant pathogen *P. syringae* was noted previously⁵. Similarly, the *P. putida* genome contains genes for even more transporters than *P. aeruginosa*, however they appear to be strongly biased toward the transport of amino acids and aromatic compounds³², both of which are likely to be found in the environment occupied by this organism. Finally, the similarity of *P. aeruginosa* secreted factors and proteins, that make up various specialized secretion pathways to those of *P. syringae* and *P. putida* appears to show less conservation. This is very likely a reflection of these species' potential interaction with a possible host in a disease setting. *P. putida* is not a pathogen and it lacks a number of toxins that target specific intracellular components of eukaryotic cells such as the components and substrates of a type III secretion pathway. In contrast, *P. syringae* not only expresses a type III secretion system, but has an unusually large number of proteins that utilize this targeting mechanism for direct translocation into host cells during infection. The type II secretion pathway, which is found in all three *Pseudomonas* species, is dedicated to secretion of degradative enzymes in *P. putida*. However, in *P. aeruginosa* and *P. syringae* these enzymes often have specific virulence promoting roles in host-pathogen interactions, such as the *P. aeruginosa* exotoxin A or the *P. syringae* pectate lyase.

Between 60 to 65% of the hypothetical unclassified and unknown genes of *P. aeruginosa* have homologues in the genomes of the other two sequenced members of this genus. The significance of this relatively underrepresented group is very likely in genes that have undergone a more recent evolution and relates to the adaptation of each species to specific environmental niches. These genes may encode proteins of entirely novel biological functions, or

alternatively, they may act in concert with the more conserved core components, but in a more customized fashion depending on the particular need of the organism.

4. PHYSICAL PROPERTIES OF THE GENOME

4.1. Genome Size

Early estimates of the size of the *P. aeruginosa* strain PAO1 genome as well as two other related strains ranged from 5,933 to 5,942 kb. Genome size was originally measured using macrorestriction analysis, employing pulsed-field gel electrophoresis to determine the sum of fragments generated by digestions with one or two restriction enzymes^{37, 42, 43}. Using similar methods, further surveys of other clinical and environment isolates showed the genome size of the majority of strains of *P. aeruginosa* ranged from 6,345 to 6,606 kb^{44, 45, 47}. The subsequent genome sequencing of *P. aeruginosa* PAO1 determined that the actual size of the single circular chromosome is 6,264,403 bp. Given this information and that the method of macrorestriction analysis has led to an underestimation of genome size because the resolution of larger fragments is limited, the average size of the *P. aeruginosa* genome is conservatively estimated to be in the range of 6.4 Mb.

4.2. GC Skew

Analyses of whole prokaryotic genome sequences have established as a common trend that G is more significantly favored over C and T is less significantly favored over A on the leading strand of replication^{29, 39, 40}. Strand bias of G over C has been described as GC skew and is quantified as $(G-C)/(G+C)$ ²³. By observing the sign of this value, the location of origin and terminus of replication can be hypothesized. In *P. aeruginosa*, the location of origin of replication has been experimentally determined⁵⁵ and in strain PAO1, the GC skew values are generally positive starting from 0 megabases (the origin) and switch sign around 2.44 Mb, suggesting the terminus of replication is located along this point^{9, 10, 15}. Interestingly, in this strain the terminus does not lie directly opposite to the origin, which would correspond to 3.15 megabases, suggesting that its displacement is not detrimental to the bacterium. Additionally, in *P. putida* KT2440, which has a large genome size of 6.2 megabases, the GC skew also switches sign asymmetrically with respect to the origin, at approximately 3,730,000 bp⁵¹. However, in *P. aeruginosa*, the terminus being asymmetrically located in relation to the origin is not a universal feature. A 2.19 megabase chromosomal inversion exists in PAO1 relative to a strain belonging to the same lineage, DSM 1707, that acted on

the *rrnA* and *rrnB* loci⁵⁰ and involved the origin of replication, displacing it from its original location directly half of the genome from the terminus. As in many other prokaryotic genomes, GC skew correlates with a greater percentage, equaling 56%, of genes located on the leading strand of strain PAO1^{9, 15}. Codon usage reflects these two biases as it is significantly different between the two strands while analysis of amino acid content of genes located on each strand show no significant bias for this variable¹⁵.

4.3. Codon Usage Patterns

4.3.1. Codon Usage Patterns of P. aeruginosa: The Effect of G+C Content. Analyses of prokaryotic genome sequences have confirmed codon usage patterns are mainly dependent on a combination of mutational biases and translational selections^{18, 41}. Codon preferences can be influenced by factors such as the level of a gene's expression, amounts of available tRNA molecules^{12, 34}, G+C content of a genome¹⁷, the strand of replication a gene is located on, or proximity to the origin of replication¹³.

Mutational bias should predictably have an effect on codon usage in *P. aeruginosa* in the form of the high %(G+C) of the genome equaling 66.56%. A study of *P. aeruginosa* codon usage in 28 genes, prior to the sequencing of the genome, showed that the mutational bias of high G+C content was a major influence on codon usage⁵². The genome sequence of *P. aeruginosa* strain PAO1 revealed that the codon preferences are extremely biased compared to the predicted frequency if the use of all codons were random. Analyses of the codon usage of the *P. aeruginosa* complete genome have shown that the majority of the codon bias is due to the high G+C content of the genome^{9, 15}. For example, *P. aeruginosa* uses the 25 most frequent codons in the genome 80 percent of the time, but if codon usage were random, these codons would be used approximately 40% of the time (raw codon usage was taken from the codon usage chart belonging to the *P. aeruginosa* PAO1 genome page on The Institute for Genomic Research website, <http://www.tigr.org>). Of the 25 most frequent codons used in *P. aeruginosa*, all but one ends in G or C, and seven are exclusively G and C. There are no codons that are comprised of only A and T. Tabulation of codon site nucleotide content for the *P. aeruginosa* genome shows that the nucleotide G is favored in the first codon position, comprising 38.6 percent of all codons and is favored against at the second codon site comprising only 20.25% of all codons used⁶. Additionally, the mean (G+C) at the first codon site, GCS₁, is 67.15%, GCS₂ is 43.4% and GCS₃ is 86.9 percent⁶. Muto and Osawa³¹ showed that a mutational bias of an organism's tendency to become more GC-rich or AT-rich is exhibited throughout the genome, but to different degrees. The authors showed that selection balanced the effects of mutational bias. For example, intergenic regions are affected the

most by mutational bias because they do not specify genes, and ribosomal RNA genes are affected the least because they are not translated and their function is essential for the organism. Additionally, they observed that the first codon position usually exhibited a higher average $\%(G+C)$ than the second codon position and the third codon position was the most affected by a skewed $\%(G+C)$. GCS_3 is the main affecter of average genomic $\%(G+C)$ because of the manner in which codons are degenerate. Synonymous codons always contain the same first two bases but contain unique third codon site nucleotides with the exception of those amino acids with more than four synonymous codons, serine, arginine, and leucine. For the mutational bias to minimally affect the amino acid content of the organism, the $\%(G+C)$ must be largely reflected in the third codon position. Other data used to support the conclusion that mutational bias is the main influence on codon usage in *P. aeruginosa* includes a common preliminary study of codon usage patterns that involves plotting the mean $\%(G+C)$ at the third codon site (GCS_3) for each gene along with the “effective number of codons” (N_c) used in that same gene⁵⁴. If codon usage patterns are only determined by the G+C content of the gene, then the trend is predicted to be located on or just below a bell-shaped curve, described by the equation $N_c = 2 + GCS_3 + \{29/[GCS_3^2 + (1 - GCS_3^2)]\}$ peaking when GCS_3 is 50%. For *P. aeruginosa* PAO1, the majority of the genes’ coordinates are interpreted to reside in the expected area, meaning that the codon usage is not very biased in the context of the G+C content⁹.

4.3.2. Codon Usage Patterns Unrelated to the G+C Content. Analysis of codon usage within a genome is usually preceded by the normalization of codon frequencies because the number of synonymous codons for different amino acids ranges from 1 to 6. A measurement known as the relative synonymous codon usage (RSCU) is produced by dividing the observed codon frequency by the expected frequency assuming all synonymous codons for an amino acid are used equally⁴⁸. In *P. aeruginosa*, not all codon usage patterns can be explained by the high $\%(G+C)$. For example, the leucine codon CUG has an RSCU value of 4.01 and is favored over another leucine codon CUC, which has an RSCU of 1.34. This unaccounted-for variation indicates there are other influences on the codon usage patterns. Common methods for examining codon usage involve computing the RSCU for each gene and subjecting these data to a type of multivariate statistical analysis known as correspondence analysis (CA)⁸. This analysis is used to analyze codon usage patterns (excluding those amino acids that are represented by one codon; methionine and tryptophan, and codons that give a termination signal) and to identify major trends responsible for variability in codon usage. CA performed with RSCU values of each gene in *P. aeruginosa* PAO1 shows that the feature that causes the largest amount of variation in codon usage, 16.9%, is high

A+T content⁹. Many of these genes are classified as being related to phage, plasmid, or transposon encoded proteins and are thought to be the result of horizontal gene transfer (HGT). A large portion of the coding sequences that are affected by this trend have unknown functions (data generated by authors, repeated analysis in the same way described by Grocock and Sharp⁹). The feature that causes the second highest amount of variation, while it is not as significant, is due to the codon preference of highly expressed genes, causing 5.2% of variation. Many of the genes that are highly affected by this trend are ribosomal proteins. Other notable proteins highly affected include PA5337 encoding the RNA polymerase omega subunit, PA2966, which is an acyl carrier protein, and many outer membrane proteins. A very small percentage of the genes affected by this trend have unknown functions. Because they are highly expressed, these genes exhibit codon usage patterns that presumably facilitate the most efficient translational event. Analysis of the codon usage of these genes highlights 19 codons representing 15 amino acids that occur more often in this set of highly expressed genes. As predicted, some of the more frequent codons correlate with those tRNA genes that are present in more copies than the other isoacceptors. For example, the leucine codon CUG is recognized by a tRNA gene located twice within the genome while the codon that it is favored over, CUC, is recognized by a tRNA that exists only as a single copy. Nine of the 19 codons have T or A in the third position and 18 out of the 19 correlate with the codons that are translationally optimal in *Escherichia coli*. The trend that results in 4.4% of variation is due to another mutational bias that affects codon usage of genes, the GC skew between the two strands of replication. This feature was also noted by comparing the GCI values (see below) of genes transcribed from the leading or the lagging strand. The χ^2 test showed that the codon usage between these two groups was significantly different, separately verifying that the GC skew has an affect on codon usage¹⁵.

4.3.3. Use of Genome Codon Index as a Measure of a Gene's Codon Usage.

A method that has been developed to compare a gene's codon usage relative to the most frequently used codons in the genome is known as the Genome Codon Index (GCI)¹⁵ and can be compared to the Codon Adaptation Index⁴⁸. GCI is calculated by taking the geometric mean of the RSCU values in each gene and dividing that by the geometric mean of the maximum genomic RSCU values for each amino acid in the gene. This gives a number that increases as the RSCU values approach the average of the genome. The average GCI in the *P. aeruginosa* genome is 0.6780. Kiewitz *et al.*¹⁵ highlighted 15 regions in the PAO1 genome with five or more consecutive genes containing unusually low GCI values (0.333–0.520), which are noted in Table 4. Low GCI values denote genes with unusual codon usage relative to the most frequently used codons used in the PAO1 genome. There are several factors that could cause

unusual GCI values for a gene. As mentioned above, the most frequent cause of variation in codon usage is due to genes with a high A+T content. Low GCI values could be due to other smaller factors listed previously such as genes that have different codon preferences because they are highly expressed or genes that are more affected by the GC skew between the strands of replication. Upon examination of the 15 islands of low GCI values, two of the regions, PA4237–PA4264 and PA4271–PA4280, appear to have different GCI values because of codon preferences resulting from translational selection (Table 4). Both these areas encode ribosomal proteins and are presumed to exhibit the codon preference of highly expressed proteins. Examination of ribosomal genes in other bacteria show highly conserved codon usage patterns as well²⁸.

5. MOSAIC STRUCTURE OF THE *P. AERUGINOSA* GENOME RESULTS FROM HORIZONTAL GENE TRANSFER

Several methods allow identification of horizontally acquired genes in sequenced genomes. Direct comparisons of gene content, using various hybridization methods or comparisons of several sequenced genomes of strains from the same species often allow identification of strain-specific segments that are obvious candidates for integration sites of genes. Alternatively such segments can represent regions of instability, where deletions can occur at unusually high frequencies. Furthermore, regional variance in %(G+C) and patterns of codon usage even in segments where %(G+C) is not significantly different from *P. aeruginosa* can be used to predict ancestral gene transfer. Other signatures of horizontal gene transfer, particularly related to genomic islands, are locations adjacent to tRNA genes and the presence of genes or remnants of genes associated with mobile genetic elements such as insertion sequences, bacteriophages, or plasmids.

5.1. Gene Content

Early indications from macrorestriction analysis using pulsed-field gel electrophoresis (PFGE) suggested that the chromosomal size of most *P. aeruginosa* isolates can fluctuate by as much as 15%, resulting in the presence of several hundred specific genes among different isolates. The availability of the genome sequence of PA14 allows for a direct comparison of two strains isolated from rather different sources. Comparison of the two genomes by BLAST analysis conservatively estimates 310 PA14 specific genes and 150 PAO1 specific genes (Frederick M. Ausubel and Jonathan M. Urbach,

personal communication). Sample sequencing of three genomes was used by Spencer *et al.*⁴⁹ to assess the occurrence of strain-specific DNA segments in the genomes of three *P. aeruginosa* strains. Based on the frequency of unique traces that cannot be aligned with PAO1 sequences or with the identified polymorphic segments and islands, the variability among strains was considerable, ranging from 8 to 15%.

Another method employed for the analysis of gene content relies on the use of DNA microarrays to identify the genes in the reference strain (used in the construction of the microarrays) that are absent from the genome of test strains. Two such studies have been carried out^{7, 53}. Wolfgang and coworkers used DNA from a collection of 18 strains of diverse origin to probe an oligonucleotide based microarray designed using the genome sequence of strain PAO1 (Figure 2), and identified a set of 5,183 core genes that were present in all of the strains tested. This number almost certainly represents an underestimate of the conserved genes, since it does not take into account sequence polymorphisms, which would not be detected using the highly stringent conditions of microarray hybridization and analysis. A similar analysis, using a spotted microarray also based on the PAO1 genome⁷, detected the presence of 89–97% of PAO1 genes in a collection of 21 isolates from environmental and clinical (cystic fibrosis and bronchiectasis) sources. Nevertheless, these studies confirmed that the large, highly conserved number of genes in *P. aeruginosa* represents an evolutionary trend of this and other free-living bacteria toward an ability to occupy a wide range of environmental niches that differs from the trend observed for certain pathogens, whose genomes are believed to be undergoing evolutionary contraction³⁰.

The strain-specific genes (those that were shown to be absent in at least one strain by the DNA hybridization analysis) were primarily represented by genes of unknown function or those associated with mobile genetic elements (plasmid, transposon, and phage-like sequences). A phylogenetic analysis based on the strain-specific gene set^{7, 53} revealed a minimal relationship among isolates from different niches, further highlighting the general diversity of the functions among the core genes. The sole exception appeared to be a cluster of strains associated with the carriage of a gene for a secreted toxin (ExoU), which formed a distinct branch of the phylogenetic tree⁵³. The extent of conservation of virulence functions is surprising, given the wide range of sources and therefore diseases that were the sources of the tested strains. Moreover, there was no clear distinction between environmental and clinical isolates. This raises the possibility that all strains of *P. aeruginosa* can express a similar armament of virulence factors including strains from natural reservoirs that have not been associated with any infection. The pathogenic potential of environmental isolates suggests that environments associated with a pathogenic lifestyle exist outside the immunocompromised human hosts.

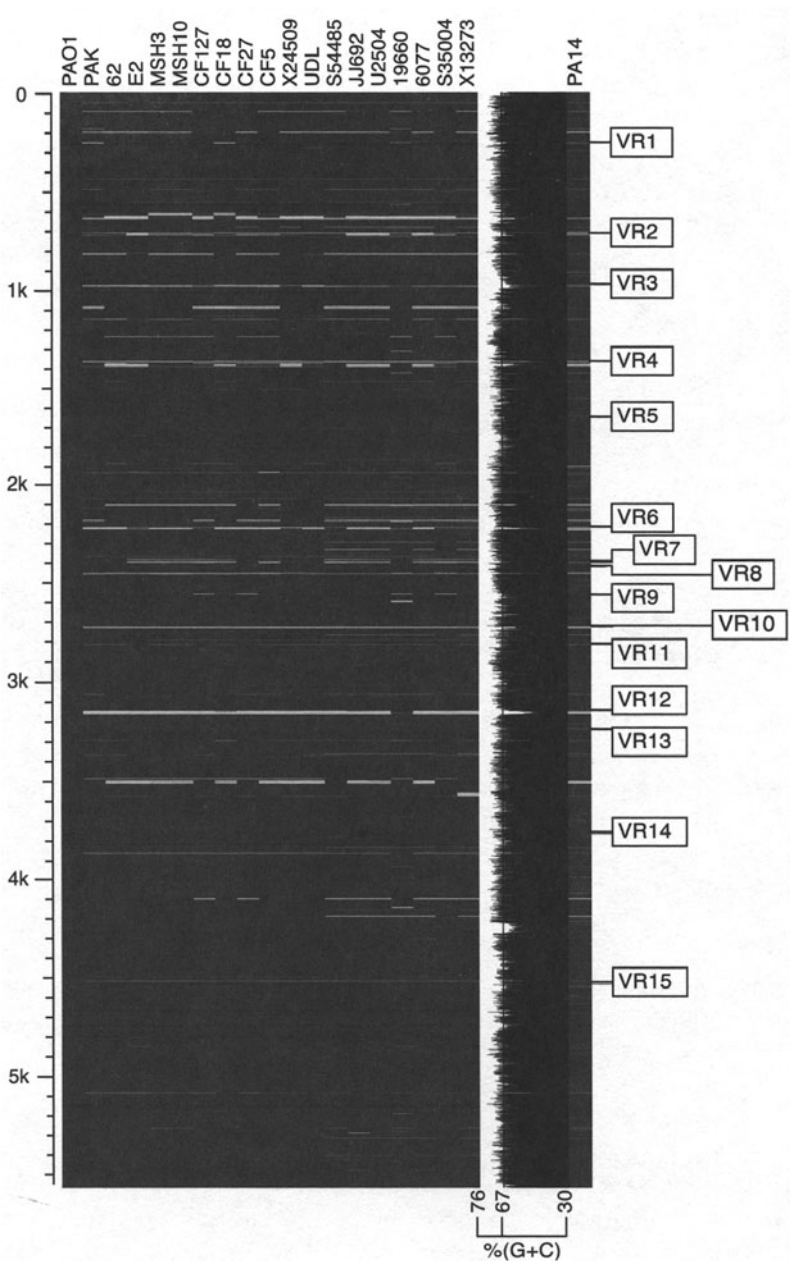


Figure 2. Interstrain comparison of *P. aeruginosa*. Results from the hybridization of 19 *P. aeruginosa* strains' (isolated from environmental and various clinical sources) chromosomal DNA to the PAO1 genome-based *P. aeruginosa*. Affymetrix GeneChip are shown above on the left (ref. [53], supplementary table 2); 5549 ORFs contained in the GeneChip are represented along the

Large polymorphic sites, indicated by an apparent absence of blocks of genes determined by microarray analysis, are not randomly distributed in the chromosome. Several such sites, relative to the PAO1 genome have been identified by the hybridization analysis using the PAO1 specific microarray^{7, 53}. An analysis from low-coverage sample sequencing of two CF and one environmental isolate identified such polymorphic regions as “gaps”⁴⁹. It is likely that some of these are genes acquired via horizontal gene transfer. Nine of these loci were present adjacent to tRNA or tmRNA genes, which suggest that the regions of variability are associated with horizontally acquired genes.

5.2. Detection of Horizontal Gene Transfer Through Analysis of G+C Content and Codon Usage Patterns

Ten genomic regions with a size greater than 3 kb and a %(G+C) less than two standard deviations below the mean were noted when the PAO1 genome sequence was published⁵⁰. These ten regions are included in the list of 15 islands with low GCI, indicating that low G+C content causes low GCI values (Table 4). The cause of most of this variation is likely due to HGT. Genes in certain organisms such as *E.coli* have often been identified as foreign or horizontally acquired because of atypical G+C content or codon usage when compared to the rest of the organism^{20, 21}. In *P. aeruginosa*, coding sequences acquired through recent horizontal transfer events can have strikingly different G+C content from the average, because of the overall skewed

Figure 2. Continued

y axis. The location of a gene can be determined by using the scale on the left-hand side in which the numbering corresponds to the location of PA gene numbers. The location of tRNA genes are indicated by the color red. Genes interpreted as being present are shown in dark blue and genes determined to be absent are colored yellow. The histogram is of the G + C content (using black bars) of each ORF included in the GeneChip and every tRNA gene located in the PAO1 genome. On the right, the nearly finished genome sequence of PA14 was compared using the BLAST algorithm to the complete list of ORFs from PAO1. PAO1 ORFs were determined to be present in PA14 if the alignment produced by the BLAST algorithm was greater than 50 percent of the entire coding sequence, produced a bit score of more than 100, and a functional ORF surrounding the alignment could be detected in PA14. Results for hybridization of PA14's chromosomal DNA were simulated based on this analysis. In addition to the present and absent parameters, two other features were included. Green represents genes present but in a location at least 10 ORFs away from its location in PAO1 and light blue represent genes that are present in the same location and duplicated at a location at least 10 ORFs away from the original location. “VR” stands for variable region and points out insertions in PA14 relative to PAO1 greater than 7 kb, and if any PAO1 homologous ORFs are located within the insertion, they make up less than 30 percent of the entire size of the insertion.

Table 3. A description of the variable regions of PA14. Variable regions in PA14 are defined in Figure 2.

Name	Left border of insertion: Name of PAO1 homologue	Right border of insertion: Name of PAO1 homologue	Size of insertion (kb) ^a	Name of insertion or known information about insertion	Percentage of the size of the insertion (kb) consisting of PAO1 homologous genes ^a	Approximate size of PAO1 region replaced (kb) ^a	tRNA located proximal to the insertion
VR1	PA0256	PA0264	35		25	10	PA0263.1 tRNA ^{Arg}
VR2	PA0714	PA0730	35		27	12	PA0729.1 tRNA ^{Gly}
VR3	PA0976	PA0988	11	PAP1-2	19	9	PA0976.1 tRNA ^{Lys}
VR4	PA1367	PA1373	16		0	8	None
VR5	PA1655	PA1656	9		28	1	None
VR6	PA2217	PA2235	35	Part of PAP1-1	27	19	None
VR7	PA2400	PA2403	18		0	18	None
VR8	PA2422	PA2423	11		0	0	None
VR9	PA2570	PA2571	44		8	0	PA2570.1 tRNA ^{Leu}
VR10	PA2729	PA2738	18		5	12	PA2736.1 tRNA ^{Pro}
VR11	PA2817	PA2820	7	Location of PAP1-2 and PAP1-3	0	2	PA2819.1-3 tRNA ^{Gly, Gly, Glu}
VR12	PA3141	PA3160	13	Probable O-antigen biosynthetic cluster	7	19	PA3139.1 tRNA ^{Asn}
VR13	5' end PA3240	3' end PA3240	21		0	0	None
VR14	PA3768	PA3770	33		11	1	None
VR15	PA4541	PA4542	108	PAP1-1	16	0	PA4541.1-3 tRNA ^{Lys, Pro, Asn}

^aValues have been rounded down to the nearest integer.

Table 4. An analysis of the relationship between G+C content and GCI.

Analysis of G+C content and GCI in all genes present in strain PAO1					
GCI ^a	Mean	0.68	%(G+C)	Mean	66.66
	First quartile	0.64		First quartile	64.80
	Median	0.70		Median	67.00
	Third quartile	0.74		Third quartile	69.20
	Min.	0.14		Min.	29.90
	Max.	0.90		Max.	76.20
Genes with atypical G+C content or GCI in strain PAO1 compared to the location of variable regions in strain PA14 ^b					
Gene range		%(G+C)	GCI		Site of VR
PA0256–PA0261		60.03	0.46		VR1
PA0614–PA0648		63.56	0.52		
PA0711–PA0729		66.70	0.48		VR2
PA0977–PA0995*		55.80	0.39		VR3
PA1149–PA1154*		58.17	0.41		
PA1366–PA1372*		51.30	0.33		VR4
PA1381–PA1394*		59.30	0.39		
PA2100–PA2106*		58.44	0.40		
PA2218–PA2228*		53.88	0.34		VR6
PA2456–PA2462*		53.67	0.38		
PA2668–PA2678		63.00	0.47		
PA2730–PA2738*		57.82	0.45		VR10
PA3140–PA3160*		52.41	0.34		VR12
PA4237–PA4264*		58.00	0.52		
PA4271PA4280		60.00	0.50		
Analysis of G+C content and GCI in genes present in PAGI-2 located in strain C ^c					
GCI	Mean	0.53	%(G+C)	Mean	64.61
	Median	0.54		Median	64.80
	Min.	0.28		Min.	55.70
	Max.	0.73		Max.	75.10
Analysis of G+C content and GCI in genes present in PAGI-3 located in strain SG17M ^c					
GCI	Mean	0.45	%(G+C)	Mean	59.72
	Median	0.45		Median	60.70
	Min.	0.18		Min.	38.30
	Max.	0.76		Max.	71.10

*Denotes regions of the genome that are >3 kb and contain a %(G+C) of less than 2 standard deviations below the mean. This data is originally found in Stover *et al.*⁵⁰ in the supplementary data. ^aAnalysis of %(G+C) in the PAO1 genome was performed using the mean %(G+C) values for each gene to provide a better comparison with GCI. Mean %(G+C) values for each PAO1 coding sequence were taken from Wolfgang *et al.* (ref. [53], supplementary table 2). GCI values for the PAO1 genome were found in Kiewitz *et al.*^{15, 13}. ^bRegions of unusually low GCI values were taken from Kiewitz *et al.*¹⁵. ^cGCI and %(G+C) values for each gene located in PAGI-2 or PAGI-3 were taken from Larbig *et al.*¹⁹.

G+C content. PAPI-1, a 108 kb pathogenicity island in strain PA14 has a mean (G+C) of 59.7 percent and PAPI-2, an 11 kb island also in strain PA14, has a mean %(G+C) of 56.4 percent¹¹. The O-antigen biosynthetic locus in strain PAO1 has an average (G+C) of 51.8%. There are at least 20 unique serotype strains known to exist in *P. aeruginosa*, resulting from variation at this locus. Eleven samples of this cluster representing diverse serotype strains have been sequenced and their G+C content ranges from 46 to 54%³⁸. Not surprisingly the O-antigen biosynthetic cluster in PAO1 has an unusually low GCI of 0.334.

Another region with a low G+C content and low GCI, PA0977–PA0995, that is in the *phnAB–oprL* region, has been identified as containing a genomic island (consisting of PA0977–PA0987) in PAO1¹⁴ (see Table 4). A tRNA gene is located proximal to this region immediately upstream of PA0977. These genes are known to be attachment sites for site-specific recombination allowing integration of foreign DNA. The 3' end of the tRNA gene is duplicated 8.9 kb from the original tRNA gene upstream of PA0988, which is usually the result of an integration event via site-specific recombination. A strain of *P. aeruginosa*, belonging to the clone K, has been identified that does not contain this insertion and in which the organization of the genes appears to represent how this region in PAO1 would have existed prior to an integration event. A large 106 kb plasmid, pKLL106, has been identified as integrating into this tRNA via site-specific integration in another related strain also belonging to the clone K, which presumably did not contain the island located in PAO1¹⁴. Lastly, in the ocular isolate strain 6077 and in the urinary tract infection isolate strain JJ692, another large 81 kb island replaces PA0977–PA0987⁵³ that also appears to have integrated via site-specific recombination acting on the tRNA^{Lys} gene.

It is not unusual for a region to have originated due to HGT to be “replaced” by another region in another strain. This may occur because of preferential integration at a particular location, such as sites adjacent to tRNAs. Alternatively, the integration of heterologous segments may occur by double reciprocal recombination along homologous segments flanking the particular polymorphic region³³. For example, 7 out of 15 of the variable regions described previously (Table 3) replace regions described as containing atypical GCI. Only five of those seven regions were named as regions with low G+C content. Six other variable regions may have unusual codon usage but would not have been detected from this analysis because they displace smaller regions that consist of less than five ORFs.

Not all DNA segments resulting from a horizontal gene transfer event will have a low G+C content as it may have been acquired from an organism with a G+C content similar to *P. aeruginosa*. Aside from low G+C content, codon usage may be another way to identify regions of DNA that have been recently acquired and may be reflected by a low GCI value (this is only one possible

explanation for a low GCI value and the other factors affecting codon usage must be considered). This conclusion is supported by the fact that two of the regions noted as having low GCI values but not low $\%(G+C)$, PA0614–PA0648 and PA0711–PA0729, have been described as possible bacteriophage derived regions⁵⁰. Also, two of the atypical GCI islands (PA0256–PA0261 and PA0711–PA0729) are areas that have been replaced by variable regions in PA14 and do not have unusually low $G+C$ content. Two other examples that have been included in Table 4, PAGI-2 from strain C and PAGI-3 from strain SG17M, are genomic islands that are thought to be present because of recent HGT¹⁹. PAGI-2 is 110 kb and has 99.97% nucleotide similarity to a region located in the chromosome of *Ralstonia metallidurans* CH34. PAGI-2 provides a good example of regions that have been horizontally transferred recently but do not exhibit extremely different $G+C$ content from the mean. The coding sequences in PAGI-2 have a mean $G+C$ content of 64.61%. This value compared to the mean $G+C$ content of all of the coding sequences in *P. aeruginosa*, 66.7% is not drastically different. However, the mean GCI value for the PAGI-2, 0.53, is much more different than the average GCI value in PAO1, 0.678. This difference may be explained by more subtle variations in codon usage patterns between two GC-rich prokaryotes. *Deinococcus radiodurans* R1 provides a good comparison to *P. aeruginosa* because its mean $G+C$ content is 66.5%. Differences in codon usage between the two can be quite obvious, such as for the proline codon, CCC, and the threonine codon, ACC. The RSCU values for *D. radiodurans* are 1.76 and 2.35 respectively but for *P. aeruginosa*, the RSCU values are 0.97 and 2.94 (this data was taken from the codon usage chart belonging to the *D. radiodurans* R1 genome page on The Institute for Genomic Research website, <http://www.tigr.org>). From these examples it is quite possible that the GCI values also provide a means to detect unusual codon usage patterns in genes that have been acquired through horizontal transfer.

5.3. Specific Genomic Islands

5.3.1. *P. aeruginosa* Genomic Island 1 (PAGI-1). The first systematic search leading to the detailed analysis of a genomic island in *P. aeruginosa* was reported by Liang *et al.*²². DNA probes derived from strain PAO1 were used to screen an M13 library of DNA from a *P. aeruginosa* strain isolated from a urinary tract infection (UTI). Clones that failed to hybridize to PAO1 DNA were presumed to represent unique sequences absent from the PAO1 genome. The 48,893-bp island, which appears to have portions of it widely distributed among various *P. aeruginosa* isolates, was sequenced and annotated. A survey of *P. aeruginosa* strains of diverse origins showed that a substantial fraction of these strains have integrated PAGI-1 at the same chromosomal location. In the

PAO1 genome, this site is occupied by a 6,729 segment located between 2,445,681 and 2,438,952 bp and tRNA genes are absent in this region of the *P. aeruginosa* chromosome. This region is particularly polymorphic and is part of a large 100 kb gap in the genome of a CF isolate, which was identified by sample sequencing of an isolate from an older CF patient⁴⁹.

Annotation of the sequence of PAGI-1 identified 51 ORFs. Although nearly half of the genes encoded proteins of unknown function, PAGI-1 contains two coding sequences that are both duplicated copies of PAO1 genes PA4689 and PA4690, are homologues of genes characterized as paraquat inducible and therefore, may function in the detoxification of reactive oxygen species. Additionally, the large number of dehydrogenases in this island may provide reduced cofactors for the detoxification reactions providing the PAGI-1 carrying strains with protection against oxidative damage. The island also encodes two putative insertion sequences further confirming that this segment shares some features of a mobile genetic element.

Within PAGI-1, genes specifying two putative transcriptional regulators were also identified. One of these is a homologue of a family of transcriptional activators that work in concert with the alternative sigma factor RpoN. Promoter sequences recognized by RpoN-containing RNA polymerase are readily identifiable due to a highly conserved sequence that serves as a binding site of this alternative sigma factor. However, examination of putative regulatory regions of all 51 genes in PAGI-1 did not show the presence of any RpoN-dependent promoter sequences. This finding suggests that the acquisition of PAGI-1 may influence the transcription of genes located outside of the genomic island. Interestingly, the 6,779 bp DNA segment present in PAO1 and in the genomes of several strains that is replaced in PAGI-1 containing strains also encodes transcriptional activators belonging to the LysR family. Genes encoding transcriptional regulators are commonly found among different pathogenicity islands. It is therefore tempting to speculate that the horizontal gene transfer event, involving acquisition of a block of new genes is accompanied by a large reprogramming of the regulatory network of *P. aeruginosa* through acquisition and loss of genes specifying transcriptional regulators.

The analysis of distribution of guanine and cytosine nucleotides in PAGI-1 revealed significant asymmetry, with one portion of the island having a %(G+C) of 65%, and therefore resembling that of the *P. aeruginosa* genome while the remainder was significantly lower in its %(G+C), 54.9%. It appears, from this difference in %(G+C), that the island was assembled from two ancestral components. Further supporting this statement, 35 kb of only the higher G+C content portion of this island is found in *P. putida* KT2440³². This region has the same gene order as PAGI-I and has 90% sequence similarity but does not include the first four ORFs containing the two paraquat inducible homologues.

5.3.2. *Pseudomonas aeruginosa* Genomic Islands 2 and 3 (PAGI-2 and PAGI-3). Using macrorestriction analysis of two strains belonging to a predominant lineage of *P. aeruginosa* associated with cystic fibrosis (CF) and environmental reservoirs in Germany ("clone C"), Tummeler and colleagues identified two large islands over 100 kb which are integrated at different but closely linked loci in the *P. aeruginosa* chromosome¹⁹. The integration of the islands both result in a substitution of a 2,001 bp DNA segment present in PAO1 containing a single gene of unknown function located between 3,171,531 and 3,173,531 bp of the PAO1 chromosome. This region is bordered on one side by two identical tRNA^{Gly} genes separated by 84 bp, one serving as the integration site for PAGI-2 in strain C, the other for PAGI-3 in SG17M. The %(G+C) of PAGI-2 is 64.6%, only slightly lower than that of PAO1, while the %(G+C) of PAGI-3 is significantly lower (59.8%). Interestingly, a nearly identical copy of PAGI-2 was identified during sequencing of the genome of *Ralstonia metallidurans* CH34¹⁹. The annotation of the two islands revealed a strikingly similar organization of the various ORFs within the island. Each island is composed of two parts. The first part of PAGI-2 contains 51 genes, specifically clustered on the left portion of the island that are predominantly homologues of genes in other organisms that specify heavy metal transport and modification functions. These included enzymes involved in cytochrome C biogenesis, transporter ATPase and thiol-disulfide 4 exchange proteins, and several transcriptional regulators. Sixty-two genes specifying mostly proteins of unknown function make up the remainder of the islands. Genes in PAGI-3 are similarly organized with 51 strain-specific genes and 54 genes of largely unknown function, with a substantial fraction of these being homologous to the 62 genes found at a similar location in PAGI-2. The strain-specific portion of PAGI-3 encodes determinants of metabolism, particularly associated with fatty acid, amino acid, or coenzyme synthesis or transport. This portion of the island also contains several transposons and insertion sequences. Both PAGI-2 and PAGI-3 contain several genes for transcriptional factors that may be involved in regulation of expression of genes encoded within the island or in the rest of the chromosome. The first gene of each island is similar to phage integrases. It is highly likely that the putative integrase encoded by these genes directed the site-specific insertion of each island at these particular tRNA^{Gly} loci.

Although most of the genes in the mutually homologous portion of the island specify unknown function, those few that show similarity to known genes appear to be associated with DNA repair, recombination, site-specific integration, or chromosome partitioning. Other gene of unknown function can be detected as homologues of unknown bacteriophage or plasmid-encoded genes. Therefore, this portion of the island very likely functions in mobilization and transfer of ancestral genetic elements between bacteria.

5.3.3. Integration of pKLK106 into the Chromosome of P. aeruginosa Clone K. Analysis of a strain's genome from a clone K lineage of serial isolates from a CF patient¹⁴ revealed the presence of a large integrated plasmid, pKLK106. This DNA was known to exist as a plasmid because an episomal form of this specific DNA region was also detected in other related strains. The plasmid apparently utilized one of two attachment sites in the chromosome that are part of a tRNA gene for reversible integration. This site was, specifically, the tRNA^{Lys} gene near the *pilA* locus, at 5,086,925 bp. This plasmid can also insert into the tRNA^{Lys} gene in a region near the *phnAB-oprL* region, between 1,060,356 and 1,067,817 bp. This site is apparently occupied in PAO1 by a smaller genomic island and is used for integration of different horizontally transferred DNA in PA14 and perhaps other strains as well. The tRNA^{Lys} gene near the *pilA* locus does not appear to have a genomic island integrated in PAO1, but is the same site used for reversible integration of another related plasmid (pKLC102) in clone C isolates. Both plasmids are thought to be related because they carry almost identical integrases responsible for their integration and have highly similar restriction enzyme fingerprints.

5.3.4. P. aeruginosa Pathogenicity Island I (PAPI-1). Strain PA14 carries two islands integrated at the same tRNA^{Lys} sites used by pKLK106, PAPI-1 and PAPI-2¹¹. PAPI-1 was initially identified after PA14 was subjected to transposon mutagenesis and a specific insertion showed reduced virulence in several models of infection³⁵. Subsequent sequence analysis and annotation confirmed a segment of DNA proximal to the transposon insertion with attenuated virulence was a genomic island. This island carries virulence genes and therefore is referred to as a pathogenicity island. Annotation of the 115 PAPI-1 genes within the 108-kb island revealed that a limited number of genes belonging to PAPI-I are homologous to those found in PAPI-2 and PAPI-3, including those involved in plasmid replication as well as a number of hypothetical unknown genes conserved in all three islands. The region of PAPI-1 encodes a portion of DNA homologous to a PAO1 ORF, PA0977, followed by a phage-like integrase determinant, which is 86% identical to the integrase gene found at the similar location following integration of pKLK106. This may be the enzyme that is responsible for the insertion of the PAPI-1 element at the specific site adjacent to tRNA^{Lys}. The *c.* 50 gene putative virulence repertoire includes several complete fimbrial adhesion systems and a secretion apparatus. The high level of similarity between the PAPI-1 genes and several plant pathogens, such as *Xylella fastidiosa*, *Agrobacterium tumefaciens*, *P. syringae*, and *Xanthomonas campestris* suggests that this element may have been acquired from these plant pathogens giving *P. aeruginosa* PA14 an unusually broad host range.

5.3.5. PAPI-2 and *ExoU* Island. Several *P. aeruginosa* strains carry the *exoU* gene encoding a protein with phospholipase activity secreted by the type III secretions system. In several strains analyzed, this gene appears to be located at the same site^{11, 53} (Bridget R. Kulasekara, unpublished results). Two *exoU*-containing genomic islands have been more extensively characterized in strains of distinct origin, and they are adjacent to the tRNA^{Lys} gene in the *phnA* region, which was used in strains belonging to clone K as one of the two integration sites of pCLK106. This location serves as a site of integration for a number of different genomic islands, however, there is no apparent correlation between *exoU* carriage and presence of a genomic island at this location.

The 10.7-kb pathogenicity island containing *exoU*, termed PAPI-2, is present in the genome of PA14 and was found during investigation of PAPI-1. Another island known to contain *exoU*, termed *ExoU* Island, is an 81-kb island identified as residing in the exact same location as PAPI-2 and has been found in ocular isolate, 6077, and urinary tract isolate, JJ692⁵³. Although quite different in size and gene content, these islands share several features. The *exoU* gene and its cognate chaperone gene *spcU* are located at the right side of the islands (relative to the tRNA^{Lys} gene). Each island carries an integrase gene at the opposite left end of the island (Bridget Kulasekara, unpublished results). The integrases encoded within each of the islands are highly homologous. Each island contains several genes homologous to genes within the PAO1 genome as well as a number of transposons.

5.3.6. The O-antigen Biosynthetic Locus. The diversity of O-antigens in *P. aeruginosa* is the result of the addition of unique carbohydrates to the lipid A-core polysaccharide. Twenty serotype strains in which representative members, called International Antigenic Typing System (IATS) strains, based on the reactivity of specific sera with the O-antigen, have been characterized³⁸. The determinants for the specific biosynthetic enzymes specifying the type of O-antigen are located between 3,527,428 and 3,546,926 bp within the PAO1 chromosome. In the majority of strains, the location of this site is conserved. The known exceptions are strains expressing the O15 serotype, in which the O-antigen determining genes reside at a different location. The O-antigen gene cluster is located adjacent to tRNA^{Asn}, and shows instability in at least one serotype (O5). Sequence analysis of examples from the 20 serotype strains shows the diversity is due to clusters of genes within 11 unique islands, ranging from 14 to 25 kb, while the remaining serotypes are caused by mutations within several of these islands. The various forms of this biosynthetic locus show some of the hallmarks of genomic islands. It is located adjacent to a tRNA^{Asn}, and the %(G+C) varies between 47 and 54%, well below that of the rest of the genome. Two of the islands carry insertion sequences, but there is no evidence for an integrase gene at the most obvious location near the junction

of the island with the chromosome. This configuration does not exclude the possibility that, following acquisition of DNA containing an island for a different serotype, incorporation of the determinants encoding a new serotype is mediated simply by double reciprocal recombination via the conserved flanking sequences.

5.3.7. The Glycosylation Island. All strains of *P. aeruginosa* express either type a or b flagellin sequence variants. Moreover, the a-type flagellins are glycosylated. The determinants of glycosylation of a-type flagellin are located at a distinct site in the chromosome and are absent from the strains expressing b-type flagellin². The Glycosylation Island, identified in several a-types, consists of a 14-gene cluster, located between 1,175,559 and 1,182,697 bp relative to the PAO1 chromosome and encodes a number of enzymes involved in biosynthesis of polysaccharides or their modification. In b-type flagellin strains, this island is absent, however in its place, a shorter DNA segment of 7,138 bp is found that contains three genes. Microarray analysis of an extensive collection of *P. aeruginosa* isolates' gene content reveals heterogeneity regarding the presence of the a-types' flagellin glycosylation enzymes coding sequences. A majority of the strains analyzed carry a truncated form of the island, with nine genes located at the left portion of the island retained³. Both the full-length and the truncated islands have the same boundaries relative to the PAO1 genome. This arrangement may represent another mechanism of glycosylation diversity, whereby acquisition of a full-length or truncated island leads to flagella displaying different oligosaccharides. There is no evidence of any features attributable to the Glycosylation Island existing as a remnant of a mobile genetic element, including the absence of adjacent tRNA genes, %(G+C) reflecting the rest of the genome, and relative stability of the island in the face of strong selection against expression of flagellin, which is a strong pro-inflammatory signaling molecule.

6. GENOMIC REARRANGEMENTS

One of the more frequently encountered genomic rearrangements involves inversions of blocks of DNA by recombination between highly homologous segments of DNA. The genome of the sequenced strain PAO1 differed from another strain (DSM-1707) of the same lineage by a large inversion of 2.19 Mb between two ribosomal RNA operons, *rrnA* and *rrnB*^{4,50}. It is likely that this inversion influences the *rrnA* genes and it may exert other influences by changing the location of genes relative to the origin of replication. Although it has been suggested that inversions involving the origin of replication can affect the fitness of the organism, including growth rates, this is

apparently not the case in clonal lineages of *P. aeruginosa* strains carrying inversions around *oriC*⁴.

A more extensive investigation of genomic rearrangements of strains of an apparent clonal lineage was carried out by Tummler and coworkers. Macrorestriction analysis was employed to identify a major clone (clone C) of *P. aeruginosa* that was highly represented at an aquatic environment in Germany and, additionally, lineages of this clone were isolated from a group of patients attending a local cystic fibrosis clinic⁴⁴. Using macrorestriction analysis, several types of genomic rearrangements have been detected in these strains. These include a large-scale inversion found in nearly half of the strains, which is centered around the origin of termination with recombination sites that were not the *rrn* loci⁴⁶. Other changes having a pronounced influence on chromosome structure were deletions and insertions of large blocks of DNA, resulting in a detectable variation in the size of the chromosome (6,345–6,606 kb).

One of the more striking discoveries regarding the consequences of chromosomal inversions in *P. aeruginosa* was made by Kresse *et al.*¹⁶ who analyzed several related members of the clone C lineage and described the genetic basis for chromosomal inversions involving intragenomic transposition of a resident insertion element. The strains belonging to the *P. aeruginosa* clone C carry a chromosomally integrated 106-kb plasmid containing two IS6100 sequences that may function as a composite transposon. Analysis of related strains isolated from the same apteins revealed that one of the IS6100 elements has the ability to transpose at various loci in the chromosome, creating a duplication that is the basis of a subsequent inversion. If the target of the insertion is an ORF, the inversion will disrupt the particular gene and may make the mutation permanent because reversion back to the original state would require two sequential events. The authors further identify several specific rearrangements, involving genes that have been previously associated with pathoadaptive mutations in CF isolates. Insertion of IS6100 into *wbpM* (encoding an enzyme for the biosynthesis of O-side chains of lipopolysaccharide) *pilB* (a type IV pilus biogenesis determinant) and *mutS* (encoding an enzyme responsible for mismatched DNA repair) leads to the phenotypes (LPS-rough, non-piliated, and hypermutable) previously shown to occur at high frequency in CF isolates. Another IS6100 insertion/inversion characterized in this study occurred in a homologue of the *dedA* gene, which presumably encodes a transporter of unknown specificity. It is conceivable that DedA inactivation is somewhat favored in the milieu of the CF lung. Since IS6100 is a highly promiscuous transposon, its insertion into a second site within a nonessential gene can lead to inversion of any segments of the chromosome. Indeed the four inversions identified in this study range in size from several kilobase to 4,085 kb. A more thorough analysis of genomes carrying various insertion sequences may reveal a common mechanism of inversion using similar intrachromosomal transposition mechanisms.

7. CONCLUSION

There is hardly a single area of *P. aeruginosa* research that has not been impacted by the availability of the genetic blueprint of this organism. It is inconceivable to imagine that today, it would be possible to carry out cutting-edge work in this complex organism without the prior knowledge of its genetic repertoire. Moreover, the available sequence information fueled rapid development of additional tools of functional genomics, most notably a genome-wide microarray, that have been successfully applied toward studies of gene content and transcriptional profiling in a variety of environments and genetic backgrounds. Two observations are highly relevant to this work; neither one of them is necessarily surprising. The number and variety of genes that allow *P. aeruginosa* to thrive in a wide range of environments places this organism as being truly ubiquitous, arguably one of the most developed bacterial species in terms of range of habitats it can occupy. Second, it is apparent that the majority of the genes in this unusually large genome are conserved among all strains, however, fine-tuning of the genome can take place through horizontal gene transfer. It is the interplay between the products of the core genes and the functions of novel genes that define specific strains of this organism and represents a new frontier in research regarding the evolutionary biology of *P. aeruginosa*. No less important is the recognition that this organism is still responsible for some of the more serious, life-threatening infections and is resistant to the most widely used antibiotics. The genome sequence may provide the majority of clues that will be necessary for the development of novel approaches toward therapy of human *P. aeruginosa* infections.

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GENOMIC FEATURES OF *PSEUDOMONAS PUTIDA* STRAIN KT2440

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1. INTRODUCTION

Pseudomonas putida strains are rapidly growing bacteria, frequently isolated from most temperate soils and waters, particularly polluted soils. They are nutritional opportunists *par excellence* and a paradigm of metabolically versatile microorganisms that recycle organic wastes in aerobic and microaerophilic compartments of the environment, and that plays a key role in the maintenance of environmental quality. *P. putida* strain KT2440^{2, 53} is probably the best-characterized saprophytic laboratory Pseudomonad that has retained its ability to survive and function in the environment. The bacterium is a plasmid-free derivative of a toluene-degrading bacterium, originally designated *Pseudomonas arvilla* strain mt-2⁴⁶ and subsequently reclassified as *P. putida* mt-2^{43, 68}. It is the first Gram-negative soil bacterium to be certified by the Recombinant DNA Advisory Committee (RAC) of the United States National Institutes of Health as the host strain of a host-vector biosafety (HV1) system for gene cloning in Gram-negative soil bacteria²¹. An extensive spectrum of versatile genetic tools, in particular mini-transposons and tools based on these, have been developed for its analysis, manipulation and use as

a host for cloned genes from other soil organisms^{12, 13, 35, 41}. KT2440 is being exploited in the development of a variety of biotechnological applications, including the design of new catabolic pathways for pollutants^{19, 51, 56}, the production by biocatalysis of intermediates, including chiral synthons for chemical syntheses⁷², and quality improvement of fossil fuels, for example by desulphurization²⁴. KT2440 is also able to colonize the rhizosphere of a variety of crop plants, such as corn, wheat, strawberry, sugar cane and spinach²⁰, and is being used to develop new biopesticides and plant growth promoters that function in the plant rhizosphere.

The sequencing of the KT2440 genome by a German–American consortium⁴⁴, and comparisons of the genome sequences of KT2440, *P. aeruginosa* strain PAO1 and *P. syringae* strain DC3000, have provided significant new insights into the biology of this paradigm of an important and ubiquitous group of soil bacteria, and the underlying genomic basis of its biosafety features, and have further increased the utility of this model laboratory organism and its biotechnological applications.

2. GLOBAL GENOME FEATURES

The genome of strain KT2440 consists of a single circular chromosome of 6,181,863 base pairs (bp), whose G+C content varies between 43% and 69% (windows of 4 kbp), and has a mean value of 61.6%. The G+C content exhibits a Gaussian-like distribution with a maximum at 63.3% and a skew towards lower values. This skew mostly results from gene islands, phage genome sequence and transposons (see below) and is even more evident in the frequency distribution of tetranucleotide sequences (Figure 1). In this respect, the spatial tetranucleotide composition of the *P. putida* genome is intermediate between the homogeneous *P. aeruginosa* strain PAO1 genome, which contains only a few small islands, and the *P. syringae* strain DC3000 genome, which exhibits a bipartite distribution (Figure 1).

In most prokaryotic genomes, the leading strand for DNA replication is rich in G and the lagging strand is rich in C, such that the origin (*ori*) and the terminus of replication (*ter*) are indicated by the change of the GC skew along the chromosome. The *ter* locus in *P. putida* KT2440 is asymmetrically located with respect to the *ori* at $3,730,000 \pm 10,000$ bp. GC skew is similar in *P. aeruginosa* and in *P. putida* (leading strand 0.00322 ± 0.0014 ; lagging strand -0.0339 ± 0.0014 ; mean \pm variance). Oligo(C) and oligo(G), which predispose to the A-DNA conformation, are strongly underrepresented in the G+C-rich *P. putida* and *P. aeruginosa* genomes, suggesting that stretches of A-DNA are counterselected. A similar pattern has also been observed in other G+C rich genomes, such as those of the *Actinobacteria*, alpha-, beta- and

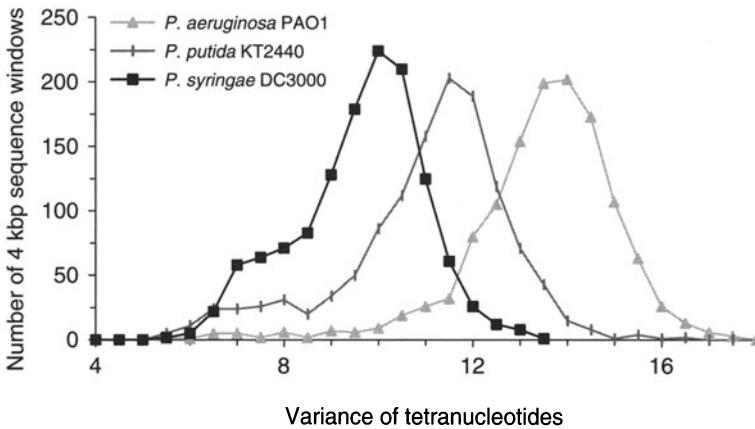


Figure 1. Distribution of tetranucleotide variances (calculated in windows of 4 kbp) in the genomes of *P. putida* strain KT2440, *P. aeruginosa* strain PAO1 and *P. syringae* strain DC3000.

numerous gamma-*Proteobacteria*. Among the most overrepresented oligonucleotides, we found an octomer (TGCTGCTG/CAGCAGCA) that is almost exclusively located within predicted genes and in a single reading frame encoding the hydrophobic tripeptide (M|V|L)LL. The high abundance of leucine specified by the *P. putida* genome (12%) as well as the high frequency of the leucine codon CUG (7.5%, with a Relative Synonymous Codon Usage (RSCU) equal to 4.07), may underlie the overrepresentation of this octameric sequence. Further prominent examples of frequent oligonucleotide words are simple repeats of the hexamer (AAGATC)_n (6 ≤ n ≤ 15) located in hypothetical genes adjacent to transposase genes and the *P. putida*-specific oligonucleotides 5'-TACCTGTGGGAGCG, 5'-GGAGCGGCCTTGTGTCGCGAT and 5'-TGT(AG)CCGGCCTCTTCGC found both in coding sequences and intergenic regions. The two latter 'words' are related to a 35 bp species-specific repetitive extragenic palindromic (REP) sequence^{1, 67}.

Codon usage in bacteria is subject to genomic and translational constraints. We calculated the genome codon index (GCI) and the codon adaptation index (CAI) for all genes of the *P. putida* KT2440 and *P. aeruginosa* PAO1 chromosomes, whereby in the case of CAI the reference gene set was either an *a priori* defined set of highly expressed genes, in analogy to *Escherichia coli*, or empirically determined by the set of genes with the strongest codon bias. The GCI compares the RSCU values of a gene with the maximum genomic RSCU values, and hence the GCI indicates the relative adaptability of the codon usage of a particular gene to the preferential codon

usage in the genome. On the other hand, the CAI indicates the relative adaptability of the codon usage of a particular gene to the codon usage of a set of highly expressed genes or a set of genes with high codon bias. Codon usage is homogeneous in *P. aeruginosa*, but heterogeneous in *P. putida*. The uniform pattern of consistently high GCI values in PAO1 is interrupted by only 15 gene islands having a codon usage atypical for *Pseudomonas*. In contrast, 105 gene islands having atypical G+C content and/or oligonucleotide signatures are scattered throughout the *P. putida* KT2440 chromosome^{66, 67}.

3. ANNOTATION OF THE *P. PUTIDA* KT2440 GENOME

The initial annotation suggested 5,420 open reading frames (ORFs), ranging in size from 90 bp (due to the arbitrary size cut-off of the applied gene-finding algorithm) to more than 26,000 bp, that would specify the proteome, seven ribosomal RNA operons, of which one occurs as a tandem (171,000–182,000) with a spacer of just a few hundred basepairs (bp), 74 tRNA genes and 2 structural RNAs. Intergenic sequences comprise 12.5% of the genome: the largest noncoding part of the genome has a size of 3,500 bp and, together with PP5238 (conserved hypothetical protein), forms a region of atypical sequence composition^{66, 67}. The second largest non-coding sequence comprises 3,494 bp and is also located within a gene island.

3.1. Pseudogenes

Eighteen genes are truncated or disrupted by a transposase or another mobile element (Table 1). These pseudogenes cluster in the chromosome region 3.5–5.5 Mb. Thirty-three other genes contain authentic frameshifts or point mutations, and most of these are also located in the 3.5–5.5 Mb region, though only a few such genes are associated with mobile elements or gene islands.

3.2. Repeats

The KT2440 genome contains 804 copies of a species-specific 35 bp REP element¹. The REP sequence consists of a central palindromic motif and characteristic residues that define the head and the tail. The consensus sequence is 5'-ccgcctcTTCGCGGGtaaaCCCGCtcctacaggg-3' (small letters: 50–89% conserved residue; capital letter: 90–100% conserved residue; palindromic region underlined). In contrast to *P. aeruginosa* and *E. coli*, in which the REP elements are typically organized in complex 'bacterial interspersed mosaic

Table I. List of truncated/disrupted genes:

ORF number	Annotation	Location	Type of truncation/ disruption
PP3399	Curl' fiber major subunit CsgA	Degeneration, conserved C-terminus	
PP4351	Flagellar protein FlhA	N-terminal 260 nucleotides	
PP1919	Thymidilate kinase	N-terminus	Truncated by gene island
PP1965		C-terminus	Interrupted by gene island (arsenate resistance)
PP1939	Formaldehyde dehydrogenase	degeneration, conserved N-terminus	
PP2521	Glutaminase family protein	N-terminus	Interrupted by ISPpu8 transposase
PP2523		C-terminus	
PP4436	Peptidase, M20/M25/M40 family	N-terminus	Interrupted by ISPpu14 transposase
PP4440		C-terminus	
PP4747	Malate/L-lactate dehydrogenase	N-terminus	Interrupted by ISPpu12 transposase
PP4744	Family protein	C-terminus	
PP3333	Conserved hypothetical protein	Degenerated, two frameshifts	
PP1327	Conserved hypothetical protein	Degenerated	
PP3408	Putative membrane protein	Degenerated, two frameshifts	
PP4023	Putative membrane protein	N-terminus	
PP4026		C-terminus	Interrupted by ISPpu12 transposase
PP3470	Transposase	Truncated	genes
			Part of gene island, adjacent to curl' proteins csgEFG
PP3673	Transposase, orfB	Degenerated	Corresponding orfA (PP3674) carries an authentic point mutation, part of gene island
PP3687	ISPpu14, transposase	Truncation	Part of gene island #76
PP4088	Rhs family protein	Degenerated	Part of gene island
PP4089	Rhs family protein	Degenerated	Part of gene island
PP4090	Rhs family protein	N-terminus	Interrupted by ISPpu12 transposase
PP4093		C-terminus	genes, part of gene island
PP4878	ISPpu9, transposase	Truncated	
PP4884	ISPpu9, transposase	Truncated	

elements', most REP elements in *P. putida* occur as single units or pairs: 225 REP sequences occur singly, 372 are located in tandem arrangements on opposite strands, and clusters of 3–5 REP sequences are found in 36, 12 and 1 case, respectively. The REP elements occur mostly in the core genome and less frequently around the terminus of replication. The role of these repeats in *P. putida* is still unknown, but do not seem to be involved in gene regulation and may serve as recognition sites for mobile elements.

3.3. Mobile Elements

The genome of *P. putida* encodes 184 proteins that are related to mobile elements, including 82 genes for transposases, 8 group II introns, a newly identified Tn7-like element (PP5407–PP5404) and the previously characterized Tn4652 (PP2984–PP2964)³ (Figure 2). Three bacteriophage genomes were identified, and a phage like region encodes an R-type pyocin, a bacteriocin probably derived from a phage. The *P. syringae* DC3000 genome contains twice as many genes related to mobile elements, whereas that of *P. aeruginosa* PAO1 contains very few.

3.4. Phages

The three bacteriophage genomes are located at positions 1,738–1,778 kb, 2,586–2,626 kb and 4,371–4,428 kb, respectively. The phage 1 genome exhibits similarity to that of KT2440 phage 3 and the D3 phage of *P. aeruginosa*, encodes 55 genes (PP1532–PP1586), including a pair of killer and antidote proteins typically responsible for plasmid maintenance. The

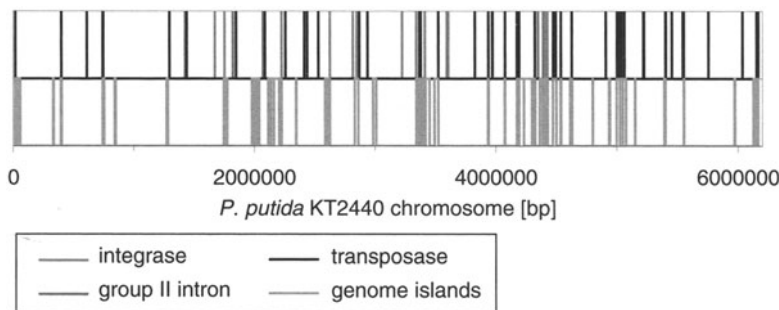


Figure 2. Localization of genes encoding integrases, transposases and group II introns, and the positions of gene islands, which are probably the result of horizontal gene transfer, in the chromosome of *P. putida* KT2440.

phage 2 genome exhibits similarity to the T7/T3 phages and encodes 32 genes (PP2266-PP2297), including a putative N-acetylmuramoyl-L-alanine amidase (PP2269), an enzyme usually involved in cell wall assembly. The genome of phage 3 is flanked by att-sites of tRNA-Cys and encodes 73 proteins (PP3849-PP3920). PP3849 is a putative haemolysin/Ca binding protein: such extracellular proteins often contribute to adhesion and virulence of bacteria. The phage genome also encodes a DNA-cytosine methyltransferase (PP3912) that may contribute to protection of the phage genome from degradation by host restriction endonucleases immediately after infection.

3.5. Genome Islands and Islets

The genome of KT2440 contains more than 100 regions of atypical oligonucleotide composition, including a number of gene islands that may have been acquired by horizontal gene transfer. Nine of these islands are larger than 20 kb and encode, besides enzymes for mobilization, functions like amino acid and opine uptake and metabolism, arsenate resistance, heavy metal resistance (e.g. copper, cadmium), oxidative stress response (peroxidase), biosynthesis of secondary metabolites, regulatory proteins and a type I restriction-modification system. These non-core functions may contribute to the fitness and versatility of *P. putida* in its natural habitat.

3.6. Transposases

The majority of 82 transposase genes cluster into eight paralogous families (ISPpu8-15) that evolved by recent duplication. The seven ISPpu10 transposases share 100% identity, even at the DNA level. Five paralogous families (ISPpu11-15) are also represented in the *P. syringae* and *P. aeruginosa* genomes. Transposition can function as a molecular switch: adaptation to solvent tolerance in *P. putida* strain S12 is triggered by transposon insertion into a regulatory gene, and inactivation of an ISPpu10 transposase resulted in impairment of root colonization by KT2440.

3.7. Group II Introns

Group II introns are retro-elements that self-splice via a lariat intermediate similar to that formed by nuclear spliceosomal introns and, frequently, encode a maturase for reverse transcription. The KT2440 genome contains eight group II introns that are highly conserved (98–100% at the nucleotide level), and all encode a maturase. The PP4446 gene was initially annotated as putative group II maturase but is only weakly related to the other maturase genes and lacks any flanking RNA structures.

Table 2. Metabolic categories of annotated genes.

Amino acid biosynthesis	126
Biosynthesis of cofactors, prosthetic groups and carriers	149
Fatty acid and phospholipid metabolism	112
Central intermediary metabolism	79
Energy metabolism	459
Purines, pyrimidines, nucleosides and nucleotides	65
DNA metabolism	118
Transcription	66
Protein synthesis	132
Protein fate	180
Cellular processes	361
Regulatory functions	535
Signal transduction	140
Transport and binding proteins	656
Cell envelope	327
Related to mobile elements	183
Unknown function	504
Conserved hypothetical proteins	1,039
Hypothetical proteins	600

3.8. Metabolic Categories

Table 2 lists the assignment of the identified coding sequences to the TIGR system of metabolic categories. Significant features are dealt with below.

3.9. Hypothetical Proteins

Among the 5,420 predicted genes coding for proteins, 600 have been annotated as genes encoding ‘hypothetical proteins’. This phrase indicates that, in most cases, no significant similarity to any other gene could be found. A certain fraction of these genes are probably artefacts produced by the gene finding algorithm¹⁴. A study by Skovgard *et al.*⁶⁰ estimated the fractions of false-positive gene predictions in completely sequenced genomes to be about 10–15%, which means that 500–800 genes in *P. putida* would be false positives. Features to identify artefacts include the absence of any similarity to known proteins, small size, atypical (oligo)nucleotide and amino acid composition, atypical codon usage, missing secondary structures, or ratio of synonymous to non-synonymous sites between homologous hypothetical proteins from two closely related species close to 1. To estimate the fraction of artefacts, we counted the hypothetical proteins that neither have a hit to any

protein family (PFAM, TIGRfam), nor to any cluster of orthologous groups (COG), and furthermore are smaller than the average size of 333 amino acids and have a below average G+C content. According to these criteria, maximally 268 genes (5% of all genes) are potential *in silico* artefacts. Ongoing annotation and functional studies will cure the current release from this small fraction of artefacts.

4. COMPARATIVE *PSEUDOMONAS* GENOMICS

The protein sequences of *P. putida* were compared with the proteomes of *P. syringae* strain DC3000 and *P. aeruginosa* strain PAO1. In order to identify potential orthologs, proteins that have diverged from a common ancestor after speciation, the best hits in both directions between the genomes were identified (algorithm: blastp, threshold: expect $>1\text{E-}05$, sequence identity $>30\%$, alignment length $>60\%$ of the individual proteins). In total, 2,945 and 3,143 potential orthologous pairs between *P. putida* and *P. syringae*, and between *P. putida* and *P. aeruginosa*, respectively, were identified.

Large sections of conserved gene order (synteny) were detected by comparing the genome locations of orthologs between *P. putida* and *P. aeruginosa* and between *P. putida* and *P. syringae* (Figure 3). The pair wise alignment of the genomes shows an X-type pattern indicative of symmetric inversions around the origin and terminus of replication, as has been observed before for several pairs of related genomes. The lack of synteny near the terminus of replication suggests that constraints on gene order are stronger near the origin. Experiments in the 1960s and 1970s suggested that genetic determinants of *P. putida* which are not homologous with those of *P. aeruginosa* cluster in the marker-poor region around the terminus of replication of the chromosome. Conserved gene order around the origin, but not around the terminus of replication is now confirmed by the genome sequence analysis: Genes lacking in *P. putida* are evenly distributed over the *P. aeruginosa* PAO1 chromosome, whereas genes lacking from *P. aeruginosa* are overrepresented around the terminus of replication of the *P. putida* genome. (Figure 3).

P. putida is more closely related to *P. syringae* than to *P. aeruginosa* in terms of the average levels of amino acid identity (80% vs 75%) and the levels of synteny (Figure 3). This agrees with the relative positions of the three species in the 16S rRNA phylogenetic tree. *P. putida* shares more homologs with alpha and beta proteobacteria, like *Agrobacterium tumefaciens* and *Ralstonia solanacearum* (expect $>1\text{E-}05$, sequence identity $>30\%$), than with the more closely related gamma proteobacteria, such as *Xanthomonas*, *E. coli* and *Vibrio* (Table 3).

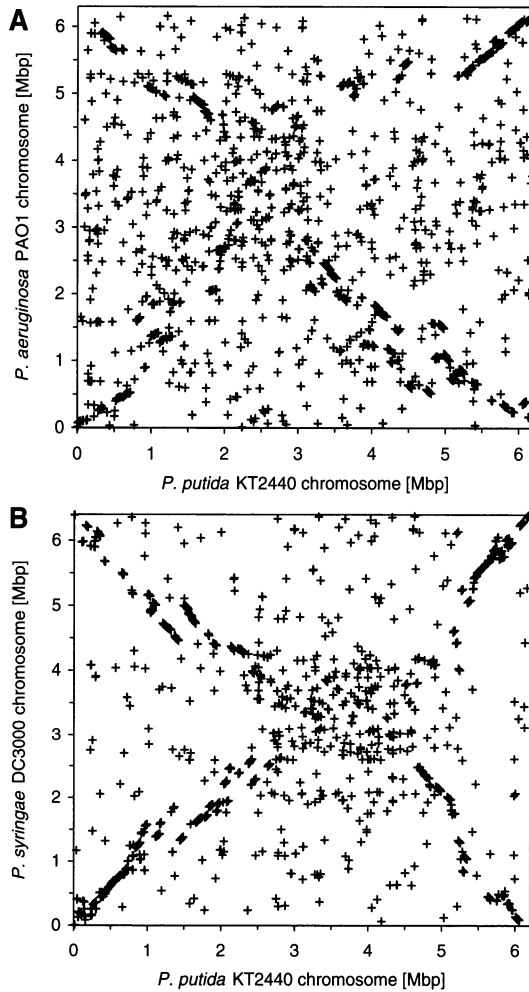


Figure 3. Comparison of the chromosomal localisation of potential orthologs in the genomes of *P. putida* KT2440 and *P. aeruginosa* PAO1 and *P. syringae* DC3000.

5. METABOLIC FUNCTIONS

5.1. Central Metabolism

KT2440 has a complete Entner–Doudoroff route for utilization of glucose and other hexoses but, like other *Pseudomonads* and rhizosymbionts, an incomplete glycolytic pathway since it lacks 6-phosphofructokinase. However,

Table 3. KT2440 genes shared with other genomes.

Organism	Phylum	No. of genes	Total protein hits	Ratio (hits/size)
<i>Pseudomonas aeruginosa</i> PAO1	Γ-proteobacteria	5,565	16,191	2.91
<i>Pseudomonas syringae</i> DC3000	Γ-proteobacteria	5,763	14,481	2.51
<i>Agrobacterium tumefaciens</i> C58 Cereon ^a	α-proteobacteria	5,296	10,501	1.98
<i>Ralstonia solanacearum</i> GMI1000	β-proteobacteria	5,116	10,039	1.96
<i>Sinorhizobium meliloti</i> 1021	α-proteobacteria	6,205	12,018	1.94
<i>Brucella melitensis</i> 16M	α-proteobacteria	3,197	5,587	1.75
<i>Escherichia coli</i> K12-MG1655	γ-proteobacteria	4,289	7,419	1.73
<i>Mesorhizobium loti</i> MAFF303099	α-proteobacteria	7,274	12,391	1.70
<i>Xanthomonas campestris</i> pv. <i>campestris</i> ATCC33913	γ-proteobacteria	4,181	7,003	1.67
<i>Xanthomonas axonopodis</i> pv. <i>citri</i> 306	γ-proteobacteria	4,312	7,139	1.66
<i>Brucella suis</i> 1330	α-proteobacteria	3,388	5,592	1.65
<i>Vibrio cholerae</i> El Tor N16961	γ-proteobacteria	3,883	6,238	1.61
<i>Caulobacter crescentus</i> CB15	α-proteobacteria	3,761	5,861	1.56
<i>Salmonella typhimurium</i> LT2 SGSC1412	γ-proteobacteria	4,553	7,082	1.56
<i>Shigella flexneri</i> 2a str. 301	γ-proteobacteria	4,180	6,423	1.54
<i>Escherichia coli</i> O157:H7 EDL933 ^a	γ-proteobacteria	5,283	8,085	1.53
<i>Yersinia pestis</i> KIM ^a	γ-proteobacteria	4,090	6,017	1.47
<i>Salmonella enterica</i> serovar <i>Typhi</i> CT18	γ-proteobacteria	4,395	6,445	1.47
<i>Shewanella oneidensis</i> MR-1	γ-proteobacteria	4,930	6,833	1.39
<i>Bacillus subtilis</i> 168	firmicutes	4,100	5,251	1.28

The analysis was carried out with the genome-vs-all alignment tool of the TIGR CMR database (similarity search on the level of protein sequences, min. aa identity: 20%, min. expect value: 1E-05).
^aThe very closely related strains *Agrobacterium tumefaciens* C58 UWash, *E. coli* O157:H7 VT2-Sakai and *Yersinia pestis* CO92, respectively, were not considered in this analysis.

it has genes coding for a fructose-1,6-biphosphatase (*fbp*; PP5040) and for glucose-6-phosphate isomerase (*pgi*; two copies: PP1808 and PP4701), which specify the initial steps of gluconeogenesis, up to glucose-6-phosphate, as well as in EPS biosynthesis. Also, like PAO1, it lacks genes for aldose-1-epimerase (*galM*) and glucose-1-phosphatase (*agp*). Glucose is thus converted to glyceraldehyde-3-phosphate and pyruvate via the Entner–Doudoroff pathway, in which 6-phosphogluconate is a key intermediate. The patchy distribution of the various gene clusters coding for the enzymes of the initial stages of glucose utilization, and the presence of different regulators in these clusters suggests a complex interplay of regulatory mechanisms and different control loops.

All genes for enzymes of the pentose phosphate pathway, the tricarboxylic acid cycle, the glyoxylate shunt, and the oxidative and electron transport chain are present. ATP synthesis is driven by the resulting chemio-osmotic gradient and occurs by an F-type ATP synthase. Analysis of the genome

suggests that carbon and energy can be derived from the metabolism of a range of compounds including acetoin, fructose, butyrate, betaine, glucose, gluconate, glutarate, glycerol, hydantoin, lactate, malate, mannose, ribose, sorbate and sucrose, among others, and that nitrogen and energy can be derived from the catabolism of the amino acids alanine, arginine, aspartate, asparagine, glutamine, glutamate, glycine, histidine, leucine, isoleucine, methionine, phenylalanine, serine, valine, lysine, proline and hydroxyproline.

Genes for at least two β -oxidation pathways are present, as are determinants for the synthesis of an extensive range of cofactors, redox proteins and prosthetic groups, including biotin, folic acid, ubiquinone, pyochelin, pantothenate, coenzyme A, ubiquinone, glutathione, thioredoxin, riboflavin, FMN, FAD, NAD, NADP, porphyrin, thiamin, cobalamin, pyridoxal 5'-phosphate, tetrahydrofolate, lipoate and a large number of determinants for 2Fe-2S and 4Fe-4S clusters. In many cases, the genes encoding for the enzymes involved are not clustered together in the genome. Interestingly, although KT2440 is not known to thrive under anaerobic conditions, nor is able to denitrify, it has several determinants characteristic of metabolism under low oxygen tension, including *inter alia* those for two oxygen-independent coproporphyrinogen III oxidase genes (PP0141, PP5101), a nitrite reductase complex (*nirB* and *nirD*, PP1705-1704), and several determinants of enzymes typical of fermentative metabolism, such as D-lactate dehydrogenase (*ldhA*, PP1649, which is clustered with *gacS* (PP1650) coding for a major histidine kinase/response regulator), phosphotransacetylase (*pta*; PP0774), formaldehyde dehydrogenase (*fdhA*, PP4960, PP0328, PP3970, plus a truncated copy PP1939), and an acetoin gene cluster (PP0550-0556, which includes a 2,3-butanediol dehydrogenase). Figure 4 gives an overview of KT2440 metabolic and transport capabilities.

5.2. Peripheral Metabolism

Strains of *P. putida* metabolize a variety of unusual and sometimes toxic compounds, including chemically stable aromatic xenobiotics³³, and are frequently the predominant microbes isolated from polluted environments. A number of the corresponding catabolic pathways, for example those for toluene and xylenes^{69, 71-73}, (see ref. [27] for the sequence analysis of the TOL plasmid of *P. putida* mt-2), phenols⁷⁰, naphthalene¹⁸, camphor⁵⁵, biphenyls⁶¹ and other chloraromatics⁵⁴ tend to be encoded by transmissible, broad host range catabolic plasmids that readily transfer between bacteria, and/or by catabolic transposons, that readily transfer within the cell from plasmid to chromosomal locations. The location of catabolic determinants on mobile

elements that can move horizontally among Pseudomonads and related Proteobacteria, provides such bacteria with considerable evolutionary potential in terms of the metabolic capacities they can acquire to exploit new nutritional opportunities³².

Other pathways for the aerobic degradation of organic compounds such as styrene, phenylacetic acid, eugenol, dibenzodioxins, nitriles, some haloaromatics, polyaromatics and nitroaromatics, are often encoded by chromosomal determinants (see, e.g. <http://umbbd.ahc.umn.edu/> and links therein; see refs [50], [54]). KT2440 lacks catabolic plasmids, but is known to have a chromosomal pathway for the degradation of benzoate, via catechol and 3-oxoadipate. Genome analysis of KT2440 revealed genetic determinants of putative enzymes able to transform a variety of other aromatic compounds, including lignin-derived compounds such as ferulate, coniferyl- and coumaryl alcohols, aldehydes and acids, vanillate, p-coumarate, p-hydroxybenzoate and protocatechuate see ref. [36], that arise during decomposition of plant material, are abundant in the rhizosphere, and therefore constitute an important carbon pool for rhizosphere-associated microorganisms. Such compounds have also been implicated in signalling between plants and bacteria such as *Agrobacterium tumefaciens*⁶³.

A general strategy exploited by Pseudomonads for the degradation of diverse aromatic compounds is to modify their structures to channel them to common intermediates that can be funnelled into a limited number of central pathways^{10, 33}. In KT2440, for example, initial steps in the metabolism of ferulate, p-coumarate and benzoate, are mediated by different enzymes (upper pathways) but all routes ultimately converge via protocatechuate (ferulate and p-coumarate) or catechol (benzoate) on the 3-oxoadipate/ β -ketoadipate pathway. Interestingly, this pathway is found almost exclusively in soil and plant-associated microorganisms³¹, and presumably evolved in response to the large number of phenolic compounds synthesized by plants. Other relevant central pathways are the phenylacetate and homogentisate pathways. More detailed treatments of these metabolic features, including the role of the multiple oxygenases and oxidoreductases, are given in Chapters 13, 15, 16, 18, and 19 of Volume 3.

The catabolic potential of KT2440 is summarized by putative pathways and substrates presented in Table 4. However, in most cases, it is not so far possible to assign substrate specificities and, in some cases, enzymatic functions, to proteins potentially involved in biodegradation, as in most cases only similarity information is available. Therefore, for the large majority of determinants of dehydrogenases, transferases, ferredoxins, regulators and transport proteins scattered throughout the chromosome, it will be necessary to experimentally determine their cellular functions.

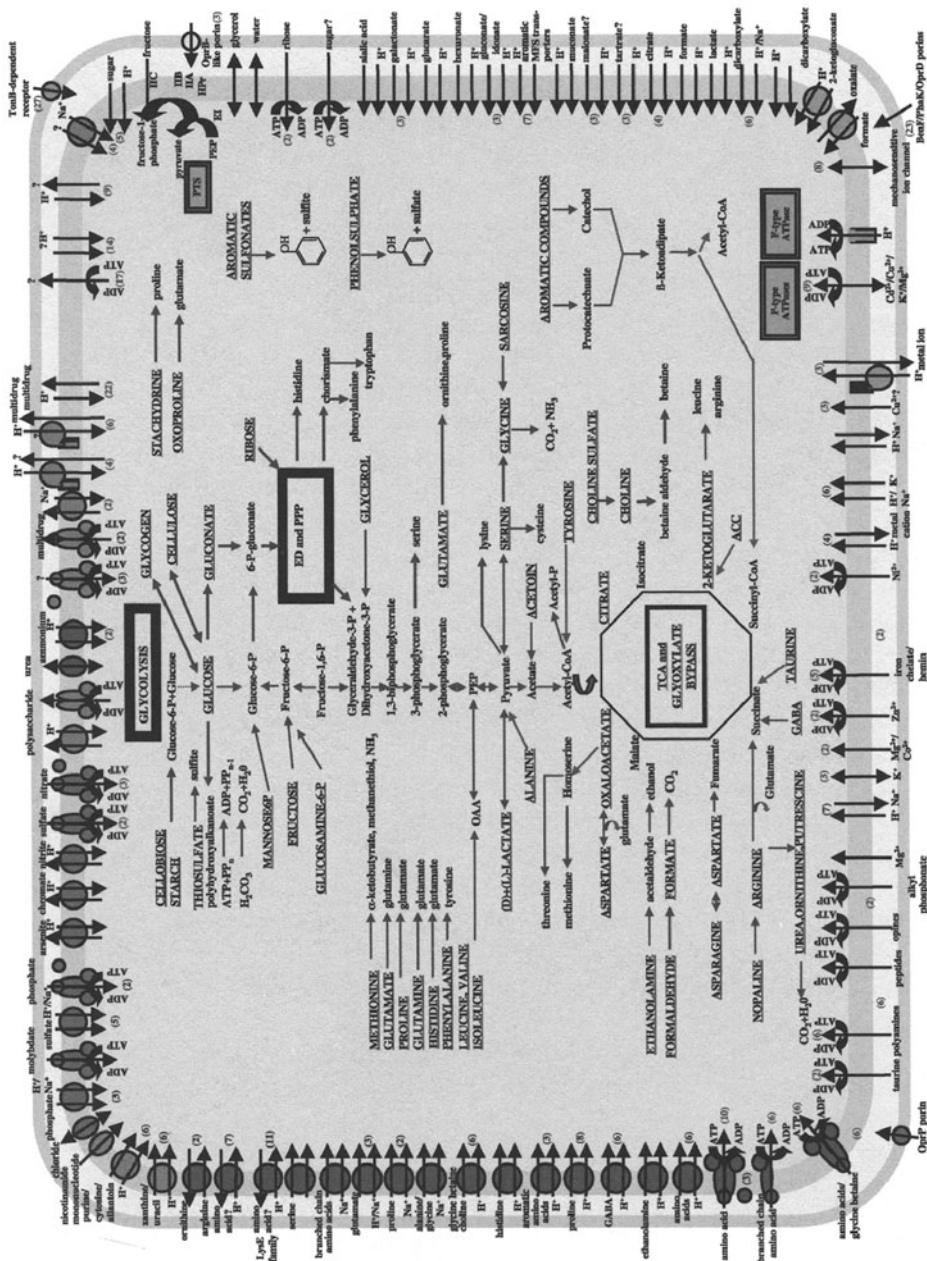


Figure 4. Overview of metabolism and transport in *P. putida* KT2440. Predicted pathways for energy production and metabolism of organic compounds are shown. Predicted transporters are grouped by substrate specificity: inorganic cations (light green), inorganic anions (Pink), carbohydrates (yellow), amino acids/peptides/amines/purines/pyrimidines and other nitrogenous compounds (red), carboxylates-, aromatic compounds and other carbon sources (dark green), water (blue), drug efflux and other (dark grey). Question marks indicate uncertainty about the substrate transported. Export or import of solutes is designated by the direction of the arrow through the transporter. The energy coupling mechanisms of the transporters are also shown: Solutes transported by channel proteins are shown with a double headed arrow; secondary transporters are shown with two arrowed lines indicating both the solute and the coupling ion; ATP-driven transporters are indicated by the ATP hydrolysis reaction; transporters with an unknown energy coupling mechanism are shown with only a single arrow. The P-type ATPases are shown with a double headed arrow to indicate they include both uptake and efflux systems. Where multiple homologous transporters with similar substrate predictions exist, the number of that type of transporter is indicated in parentheses. The outer and inner membrane are sketched in grey, the periplasmic space is indicated in light turquoise and the cytosol in dark turquoise.

Table 4. Catabolic potential of *P. putida* strain KT2440.

Putative substrate	Potential end products	Most similar to	ORF number
Aromatic/Aliphatic sulphonates	Phenol + sulfite	<i>Agrobacterium tumefaciens</i>	PP3219; PP2765
Benzoate, Toluate	Catechol (2-hydro-1,2-dihydroxybenzoate)	<i>Pseudomonas putida</i>	PP0241-PP0236
	<i>cis,cis</i> -muconic acid	<i>Pseudomonas putida</i>	PP3161-PP3164
Catechol (2-hydro-1,2-dihydroxybenzoate)		<i>Pseudomonas putida</i>	PP3713; PP3166
Coniferyl alcohol	Ferulic Acid (4-hydroxy-3-methoxycinnamate)	<i>Pseudomonas</i> sp.	PP5120
2-cyclohexen-1-one	Cyclohexanone	<i>Pseudomonas syringae</i>	PP1478; PP1254; PP2489
4-hydroxybenzoate	Protocatechuate (3,4-dihydroxybenzoate)	<i>Pseudomonas putida</i>	PP3537
Hippurate (Benzoylglycine)	Benzoate + glycine	<i>S. meliloti</i>	PP2704
Isoquinoline	isoquinolin-1(2H)-one	<i>Pseudomonas dimuta</i>	PP3622-PP3621;
		<i>S. meliloti</i>	PP2478-PP2477
Ferulic acid (4-hydroxy-3-methoxycinnamate)	Vanillin (4-hydroxy-3-methoxybenzaldehyde)	<i>Pseudomonas</i> sp.	PP3354-PP3358
Maleate	Fumarate	<i>Serratia marcescens</i>	PP3942
Phenolsulphates	Phenol + sulfate	<i>Pseudomonas aeruginosa</i>	PP3352
Phenolacetate	Phenol + acetate	<i>Pseudomonas fluorescens</i>	PP5253
Phenylalanine	Tyrosine	<i>Pseudomonas aeruginosa</i>	PP4490

Phenylacetic Acid	TCA intermediates	<i>Pseudomonas putida</i>	PP3284-PP3270
2-phenylethylamine			
phenylacetaldehyde			
phenylalkanoic acids			
3-polyhydroxybutyric acid			
phenylalkanoic acids	Polyhydroxyalkanoates (polyesters)	<i>P. putida</i> U	PP5006-PP5003
Propanediol	3-hydroxypropanol	<i>Klebsiella pneumoniae</i>	PP2803
Protocatechuate	Succinate, acetate	<i>Pseudomonas putida</i>	PP4656-PP4655; PP3952-PP3951; PP1381-PP1379
(3,4-dihydroxybenzoate)			PP3569
Quinate	5-dehydroquininate to protocatechuate	<i>Xanthomonas campestris</i>	
Taurine	Aminoacetaldehyde + succinate		PP0230; PP0169 PP4466
TNT	2-hydroxylamino-4, 6-dinitrotoluene and 4-hydroxylamino-2, 6-dinitrotoluene	<i>Pseudomonas putida</i>	PP0920
(2,4,6-trinitrotoluene)			
Vanillin (4-hydroxy-3-methoxybenzaldehyde)	Protocatechuate (3,4-dihydroxybenzoate)	<i>Pseudomonas</i> sp.	PP3357; PP3737-PP3736
Stachydrine	Proline	<i>S. meliloti</i>	PP4753-PP4752

5.3. Transporters

5.3.1. Nutrient Uptake Systems. *P. putida* KT2440 has very broad transport capabilities, with approximately 370 cytoplasmic membrane transport systems, 15% more than *P. aeruginosa*, and constituting about 12% of the whole genome. The largest family corresponds to ATP-Binding Cassette (ABC) transporter (94 paralog members), of which a significant proportion is predicted to be devoted to amino acid uptake. This is consistent with the ability for KT2440 to colonize plant roots, since root exudates are rich in amino acids, and reflects its physiological emphasis on the metabolism of amino acids and their derivatives to successfully compete in the rhizosphere. KT2440 encodes various uptake systems for osmoprotectants, compatible solutes such as glycine betaine or proline betaine, including ABC transporters for glycine (PP0871-PP0868) and proline betaine (PP294-PP296 and PP2774-PP2775), one proline betaine MFS transporter (PP2941) and six members of the choline/carnitine/betaine transporter family (PP5374, PP5061, PP3957, PP3628, PP0229 and PP2692 (putative)). The five transporters for gamma-aminobutyric acid (GABA; PP4106, PP2911, PP4756, PP2543 and PP0284), of which *P. aeruginosa* and *P. syringae* encode only one copy, may be involved in the uptake of butyric acid, which can be subsequently converted to polyhydroxyalkanoic acids (bioplastics). KT2440 also has the determinants for 11 LysE family amino acid efflux transporters (*P. aeruginosa* only has one) that presumably play a key role in preventing the accumulation of inhibitory levels of amino acids or their analogs in the cell. Also consistent with its ability to colonize plant roots, KT2440 has a predicted ABC family opine transporter (PP4453-PP4455), that has previously been described for other rhizosphere microorganisms, like *A. tumefaciens* and *S. meliloti*³⁸, and enzymes for metabolism of opines (PP4452-PP4460), suggesting that it is capable of exploiting plant-produced opines induced in the rhizosphere by other bacterial species.

Consistent with its exceptional metabolic versatility, KT2440 encodes more putative transporters for aromatic substrates than any currently sequenced microbial genome, including multiple homologs of the *Acinetobacter calcoaceticus* benzoate transporter BenK (PP3165), and of the *P. putida* 4-hydroxybenzoate transporter PcaK (PP1376, 45). In addition, KT2440 specifies 23 members of the BenF/PhaK/OprD family of porins (e.g., PP3168 [benF] or PP1383 and PP2517), outer membrane channels implicated in the uptake of aromatic substrates (e.g., PP0268 or PP2089)^{8, 49}, and a further 30 porins of unknown specificity. In addition to these transport systems, the genome contains the determinants for the import of some sugars (e.g., gluconate [PP3417] ribose [ABC transporter systems PP2459-PP2454 and PP2758-PP2761], fructose [PTS system PP0795, PP0792-PP793], organic acids (aromatic [e.g., phaK/phaJ, PP3271-PP3272], dicarboxylic and

tricarboxylic), oligopeptides (e.g., PP0878-0884), anions (taurine [PP0231-PP233], phosphate [PP0824-0827, PP2656-2659], sulfate [ABC system PP05168-5171]), plus 5 other transport proteins (PP0075, PP1407, PP0718, PP0100 and PP4112), sulfonate [PP3635-PP3637 and PP0237-PP0240], nitrate [PP0207-PP0209, PP2092-PP2094], etc.) and cations (ammonium [PP2106, PP5233], oligoelements (magnesium [PP1843, PP2645, PP3244, PP4471], copper [PP0588, PP2159, PP5394], zinc [PP117-0120] nickel [PP3343-3346], cobalt [PP0026], molybdate [PP3828-3830], etc.), potassium (PP0065, PP1200, PP3953, PP4507, PP4159-4161) (potassium transport ATPase, PP2225-PP2229 [potassium-proton multiporter], sodium [PP1132, PP1167, PP3958, PP4031, PP5066, PP5355], heme [PP4687-4689], and iron [see below]). KT2440 specifies as well three ABC transporters (PP0411-PP414, PP1482-PP1486, PP3814-PP3817) as well as several orphan periplasmic binding proteins (PP0873, PP2195, PP3147, PP3845, PP5341) for polyamines, which are ubiquitous polycationic compounds that play important roles in transcription and translation, ribosome assembly, cell growth, or membrane stability. Interestingly, KT2440, has only two incomplete TRAP family dicarboxylate transporters (PP1167, PP1169) compared with at least four in *P. aeruginosa*. It has also only one PTS sugar transporter (for fructose [PP0795, PP0792-PP793], see section Central Metabolism) and a PTS-like system for nitrogen (PP0950-PP0952).

5.3.2. Iron Acquisition. KT2440 produces a siderophore, pyoverdine, whose genes are clustered in three groups (PP4243-PP4246, PP4319-PP4327 and PP4219-PP4223) with an organization similar to that found in the phytoprotecting *P. fluorescens* and *P. putida* strains. Although the siderophore receptor for pyoverdine in each strain is highly specific for the siderophore the strain produces, fluorescent *Pseudomonads* have been shown to utilize siderophores produced by other strains, uptake being mediated by other ferric-siderophore receptors (see review of Cornelis & Matthijs, [7], this issue and Visca *et al.* [64]). Consistent with this, KT2440 has 29 genes, whose products are predicted to be TonB-dependent outer-membrane siderophore receptors, most of which are located within a gene cluster containing determinants for a transmembrane sensor, an ECF sigma-70 factor and a transcriptional regulator, as predicted by Visca *et al.*⁶⁴ for various prokaryotes. In some cases the siderophore receptors are clustered with determinants for a siderophore transport system and/or siderophore biosynthesis. The products specified by ECF sigma-70 factors regulate expression of the receptor proteins according to iron availability³⁹.

5.3.3. Efflux Systems. KT2440 has a large number of determinants for active efflux systems for metals (*inter alia* arsenite [PP1929, PP2717], copper [PP5378-PP5379 and PP2204-PP2205], cadmium [PP0041-PP0045,

PP2408–2411, PP5139], chromate [PP2556] and cyanate [PP0970, PP3751]) organic solvents (toluene [PP0960–PP0958, PP1385–PP1386]), paraquat (two paralogous sets: PP2576–PP2577 and PP0598–PP0599), antibiotics (e.g., penicillin PP0606), and, interestingly, an efflux system for the export of fusaric acid (PP1266–PP1263), a common fungal toxin produced by phytopathogens, such as *Fusarium oxysporum*⁵⁸. Most heavy metal ion transporters are encoded within two large gene islands (pos. 16–58 kbp and pos. 6,128–6,169 kbp) near the origin of replication. These include a P-type ATPase for Cd, one CDF and one CZC family transporter for the efflux of Co, Cd or Zn in one island as well as another CZC family transporter and a Cu P-type ATPase in the other island. The core genome encodes a third efflux system of the CZC family, a second Cu P-type ATPase and a Ni ABC transporter.

Forty-eight determinants for transporters of the Major Facilitator Superfamily (MFS, PFAM00083) were found, but, in many cases, the substrate is unknown. The same applies to the class RND efflux transporters. Some of the RND/MFP/OMF gene clusters are located adjacent to BenF/PhaK family porins, which is consistent with the notion that the physiological role of some RND efflux systems in *P. putida* is the efflux of toxic unmetabolizable or partially metabolizable catabolic intermediates of aromatic compounds (PP1386–1385, 59). At least one ABC efflux transporter shows some similarity to ABC pumps related to the efflux of organic solvents (PP0958–PP0960)³⁷. Some RND pumps seem to be TonB system dependent (PP5306–PP5308), as has been demonstrated for a solvent-tolerant *P. putida* strain²⁵. A member of the voltage-gated chloride channel family (PP3959) could be involved in the extrusion of excess chloride ions that may result from the degradation of chloro-organic compounds. A large fraction of the ABC transporters is predicted to be dedicated to the export of unknown substrates, possibly toxic compounds. Often, these genes are clustered with determinants of two-component systems, or key response regulators such as *agmR* (PP2665), which is involved in the regulation multiple pathways, including those for the metabolism of medium-chain-length alcohols (cluster PP2672–PP2682).

6. MONITORING AND SENSING THE ENVIRONMENT

6.1. Regulation and Signal Transduction

Almost 10% of the genes in the KT2440 genome encode products involved in signal transduction and gene regulation, which reflects the evolutionary emphasis of this bacterium on monitoring and responding to a large number of environmental signals. Common transcription factors include the sigma factors SigX, RpoD (sigma-70), RpoN (sigma-54), RpoS (sigma-38),

RpoH (sigma-32), FliA (sigma-27) and AlgT (sigma-22, homologous to RpoE in *E. coli*). Both *P. putida* 2440 and *P. aeruginosa* specify 22 sigma-54 dependent transcriptional regulators, which is considerably more than those in habitat-related microorganisms like *M. loti* (13), *S. meliloti* (12), *A. tumefaciens* (9), *R. solanacearum* (8) and *C. crescentus* (5), and far more than most other prokaryotes sequenced to date. Interestingly, *P. syringae* DC3000, a plant pathogen, has 23 (see CMR at www.tigr.org). Many of the sigma-54-dependent regulators in KT2440 appear to belong to two-component systems and possess a domain that can be phosphorylated by a sensor-kinase protein in the N-terminal section. The response regulator receiver domain family (PF00072), with 94 members is the second largest paralog family in the genome. This reflects the large number of determinants (67) for two-component signal transduction systems. The genome also contains a large number (19) of genes of extracytoplasmatic function (ECF) sigma-70 factors, many of which clustered with sensing or transport genes (see ref. [39] and Chapters 11 and 12 of Volume II).

The majority of signal transduction and regulatory genes of KT2440 (450, 93%) share homology with regulators in *P. aeruginosa* PAO1. Of the 32 regulatory elements present in the *P. putida* genome that are not shared with *P. aeruginosa*, 12 are most similar to regulators of the soil bacteria *A. tumefaciens*, *S. meliloti*, and *M. loti*. Most of these 32 elements are associated with metabolic pathways or phage regions unique to KT2440, and include the PhaN and PhaM transcriptional regulators, which are components of the phenylacetyl-coenzyme A catabolon absent from *P. aeruginosa*, a LysR type regulator that flanks a novel polysaccharide biosynthesis operon in *P. putida* (PP3143), and a PrtR transmembrane regulator that may be involved in the regulation of extracellular protease activity (5). Of the other *P. putida* unique regulators, six are associated with prophage. KT2440 also encodes 22 members of the Cro/CI family that includes transcription factors specified by bacteriophages and genome islands.

The LysR transcriptional regulator family (PF00126) is the largest paralog group in the KT2440 genome (110 members), which is a remarkably high value for prokaryotes (normal range between 2 and 12) and only comparable to the situation in *P. aeruginosa* (122), *P. fluorescens* (estimated 127), *S. meliloti* (86), *M. loti* (85), *A. tumefaciens* (75), *P. syringae* (estimated 67) and *E. coli* strains (45–60) (Table 5). Although LysR type regulators are associated with the regulation of diverse functions, they play a central role in the activation of the expression of enzymes and proteins involved in aromatic metabolism, such as in protocatechuate (PcaQ, PP1713) and catechol catabolism (CatR, PP3716) and of other soil/plant related functions. AraC transcriptional regulators (PF00165), which control processes such as carbon metabolism, stress response and pathogenesis, with 40 members, are also highly represented in the KT2440 genome, for example, the PobR (PP3538, polyhydroxybenzoate metabolism)

Table V - Regulatory motifs in various prokaryotes

Regulatory motif	<i>P. putida</i>	<i>P. aeruginosa</i>		<i>S. melilloti</i>	<i>M. loti</i>	<i>A. tumefaciens</i>	<i>C. crescentus</i>
	KT2440	PAO1					
Adenylate and Guanylate cyclase catalytic domain	0	1		25	9	3	0
Adenylate cydase, class-I	1	1		0	0	0	0
AsnC (Irp) family	13	9		15	22	14	8
Autoinducer synthetase	0	2		1	4	1	0
Bacterial regulatory helix-turn-helix proteins, araC family	40	63		38	43	36	13
Bacterial regulatory helix-turn-helix proteins, lysR family	110	122		95	85	75	12
Bacterial regulatory proteins, arsR family	7	5		17	24	14	7
Bacterial regulatory proteins, crp family	6	8		12	18	13	5
Bacterial regulatory proteins, deoR family	2	4		8	14	7	2
Bacterial regulatory proteins, Fis family	19	21		9	12	8	7
Bacterial regulatory proteins, gntR family	31	29		56	55	48	12
Bacterial regulatory proteins, lacI family	3	6		29	18	22	13
Bacterial regulatory proteins, luxR family	19	32		24	29	23	5
Bacterial regulatory proteins, merR family	10	8		7	6	7	3
Bacterial regulatory proteins, tetR family	26	42		28	47	32	30
Bacterial transcriptional regulator	7	9		9	7	8	0
CarD-like transcriptional regulator	0	0		1	1	1	1
Cold-shock DNA-binding domain	7	6		8	10	6	4
Ferric uptake regulator family	2	2		3	2	3	2

GGDEF domain	36	33	17	32	29	11
Helix-turn-helix domain, rpiR family	3	3	8	6	5	1
His Kinase A (phosphoacceptor) domain	58	55	33	43	40	44
Histidine Kinase-, DNA gyrase B-, phytochrome-like ATPase	71	67	43	57	50	56
LuxS protein	0	0	0	0	0	0
MarR family	7	17	22	32	27	9
Nitrogen regulatory protein P-II	1	1	2	2	2	3
Response regulator receiver domain	94	91	63	69	64	71
ROK family	0	0	10	7	10	3
Sigma-54 factors family	1	1	1	2	1	1
Sigma-54 interaction domain	22	22	12	13	9	5
Sigma-70 factor	4	9	4	7	2	3
Sigma-70 factor (ECF subfamily)	19	17	11	19	11	13
SIS domain	6	6	13	13	6	5
Transcriptional regulatory protein, C terminal	27	24	18	28	22	12
Total genes in organism	5420	5565	6206	7281	5420	3737
Genome size(Mb)	6.2	6.3	6.68	7.59	5.68	4.01
Total regulatory motifs	694	716	642	736	599	361
Distinct regulatory genes	535	549	539	603	486	257
% regulatory genes	9.87	9.87	8.69	8.28	8.97	6.88

and BenR (PP3159, benzoate catabolism). The KT2440 genome contains a wide diversity of other regulatory gene families, such as *tetR* (26 members, which often regulate expression of resistance to hydrophobic antibiotics and detergents), *asnC* (13), *gntR* (31), *lacI* (3), *luxR* (19), *Cro/CI* (27), *merR* (10), *marR* (7) and *fis* family (19). There are 34 genes for proteins containing a particular family of helix-turn-helix domains of the *fis* type (TIGRFAM01199, see CMR, www.tigr.org), and that are frequently associated with sigma-54 transcription factor domains and other transcriptional regulators.

The relative paucity in KT2440 (7), *P. fluorescens* (5), *P. aeruginosa* (5) and *R. solanacearum* (6), compared with much higher numbers in *M. loti* (24), *S. meliloti* (17) and *A. tumefaciens* (14), of *arsR* regulator genes, whose products generally regulate transcription of proteins involved in metal ion efflux and/or detoxification, is somewhat surprising. On the other hand, the low number of *lacI*-type transcriptional regulator genes (2 regulators plus 3 ORFs containing the corresponding PF00532 domain), whose products often recognize sugar-inducers, is consistent with the paucity of sugar-transport systems (in particular PTS) in KT2440, and with its limited spectrum of sugar and oligosaccharide utilization, compared with other prokaryotes⁴⁴. For example, KT2440 has only one ORF with a sugar isomerase domain (SIS, IPR001347), which are found in many phosphosugar isomerases and phosphosugar binding proteins, as well as in proteins that regulate the expression of genes involved in synthesis of phosphosugars, whereas *P. aeruginosa* has 6, *E. coli* 15 and rhizobia have 12–13. Also, KT2440 has no ROK family (IPR000600) genes, which code for proteins involved in sugar metabolism, unlike *E. coli* or *M. loti*, which each have 7. A more detailed description of the diverse regulatory features in Pseudomonads is presented in Chapters 7–18 of Volume 2.

Several signal transduction domains have been discovered recently, including GGDEF and EAL domains that have cyclase and phosphodiesterase activities, respectively, for the second messenger bis-(2',5')-Cyclic diguanylic acid (di-GMP). These domains were found to be causally involved in the regulation of biofilm formation. There are 39 proteins of *P. putida* that possess a GGDEF domain, 19 of which have been classified as 'unknown'. Another 21 proteins carry an EAL domain, 10 of which have been classified as 'unknown' or 'hypothetical'. A thorough characterization of these proteins will be highly instructive in the effort to understand signal transduction pathways of *P. putida*.

6.2. Stress Responses

Soil bacteria like pseudomonads are continually confronted with diverse changes in environmental parameters, and biological stresses such as osmotic and pH stresses, desiccation, suboptimal growth temperatures, and toxic chemicals. In order to monitor its environment for such changes and stresses,

and to adjust cellular physiology to compensate for them, KT2440 has a large number of genes coding for universal stress proteins¹⁰, cold shock proteins⁷, small heat shock proteins⁵, and stringent response-starvation-related proteins¹⁵. Many of these¹³ are glutathione-S-transferases, which are often involved in the detoxification of xenobiotics and heavy metals^{23, 65} and thereby contribute to microbial tolerance of noxious chemical species in their environments. The numbers of determinants of such stress proteins are substantially lower in most other genomes sequenced thus far.

Cold shock proteins, such as the CspA family proteins⁷³, are widely distributed among bacteria⁶², are immediately expressed after a temperature downshift, and mainly act as chaperones for DNA or RNA. Cold shock acclimation proteins exhibiting high expression levels at low temperatures have been identified in *Pseudomonas* species and may be responsible for their psychrotrophic phenotype^{28, 42}. There are in the KT2440 genome six determinants of proteins having a cold shock domain (csd), including two CspA-like proteins (PP0961, PP1522), one CspD ortholog (PP4010) and three cold acclimation proteins.

The first line of defence of plants against 'intruders' is the production of oxygen radicals. Plant-associated microorganisms, both pathogens and symbionts/mutualists, must therefore cope with oxidative stress. The genome of KT2440, like those of other plant-associated microorganisms, has many determinants of proteins associated with responses to oxidative stress, including superoxide dismutases, catalases, (betaine-) aldehyde dehydrogenases, etc. In some cases, the regulation of these genes is iron-dependent and coupled to that of iron-binding proteins such as bacterioferritin (*bfr*).

A major stress factor in many terrestrial habitats is oscillation in water availability. Studies of water stress in biological systems have traditionally focused on osmotic stress, which influences cell turgor, and in turn affects cellular processes like transport, cell growth, membrane integrity and regulation of cytosol concentration. However, in a recent study, Hallsworth *et al.*³⁰ showed that chaotropic solutes, which freely traverse cell membranes and therefore do not affect cell turgor, have a profound effect on water activity, and hence the stability and activity of hydrated cellular macromolecules and organelles, and to induce a water stress response in KT2440. This response was characterized by the up-regulation of cellular functions involved in protein and membrane stability, and the associated requirement for increase protein synthesis and energy generation: Chaperones, proteins involved in lipid metabolism, membrane structure, protein synthesis and energy metabolism, detoxification proteins. Since many environmental pollutants are chaotropic, and thus induce water stress even under conditions of high water availability, the ability of *P. putida* to thrive in polluted environments implies a well-developed ability to tolerate chaotrope-induced water stress.

6.3. Chemotaxis

Chemotaxis enables bacteria to spatially position themselves in physico-chemical gradients in their environments, and to translocate themselves in response to changes in environmental parameters. Depending on the parameter monitored, bacteria will respond by either swimming towards attractants or retreating from repellants. The membrane-spanning methyl-accepting chemotaxis proteins (MCP) monitor the environmental composition and transduce the signal via a number of receptor and transducer proteins, including CheW, CheV, CheA and CheY to the flagella apparatus, and thereby influence the mode of rotation and the swimming direction of the cell. The adaptation is mediated by the level of methylation of the MCP, which is controlled by the methyltransferase CheR and the methylesterase CheB. The activity of the transducer CheY is regulated by the phosphatase CheZ. In its natural habitats, *P. putida* is exposed to many stimuli, and its genome accordingly encodes 27 MCPs, including one for inorganic phosphate (PP0562), three for sensing amino acids (PP0320, PP1371, PP2249), as well as three aerotaxis receptors (PP2257, PP2111, PP4521) that are involved in energy sensing, movement towards oxygen, and retreating from toxic compounds. A similarity analysis of the aerotaxis receptors of *P. putida* with the two copies of *P. syringae* and the single receptor of *P. aeruginosa* revealed that only PP2111 (Aer-1) has orthologs in both of the other two species. PP2257 (Aer-2) whose function was described by Nichols and Harwood⁴⁵, seems to be the result of a unique duplication of PP4521, which clusters with the second aerotaxis receptor of *P. syringae*. Figure 5 gives an overview of the identified chemotaxis operons in the KT2440 genome: Two for flagella-mediated swimming (PP4332-PP4340, PP4392-PP4393), one for twitching motility towards chemoattractants (PP4992-PP4987), one uncharacterized chemotaxis cluster (PP1494-PP1488) that is shared with *P. aeruginosa* and *P. syringae* and one that is shared only with *P. syringae* (PP3762-PP3757).

The synthesis and function of the complex flagellar system requires the expression of more than 50 genes. A 66 kbp gene cluster encodes the genes for the flagella apparatus (PP4331-PP4397). Its organisation is highly similar to the flagella cluster found in *P. syringae*. The *P. putida* cluster is interrupted by a genome island (PP4345-4350) with atypical sequence composition that encodes a GntR family regulator, a cystathionine beta lyase, an aminotransferase and two copies of a D-alanine-D-alanine ligase, one of which is not annotated so far (pos: 4943725-4944636). The island was inserted into the *flhA* gene which exists in a full-length (PP4344) and truncated copy (PP4351). The very short hypothetical proteins PP4360 and PP4387 in the flagella cluster of PP are not conserved in any other genome and might be artefacts.

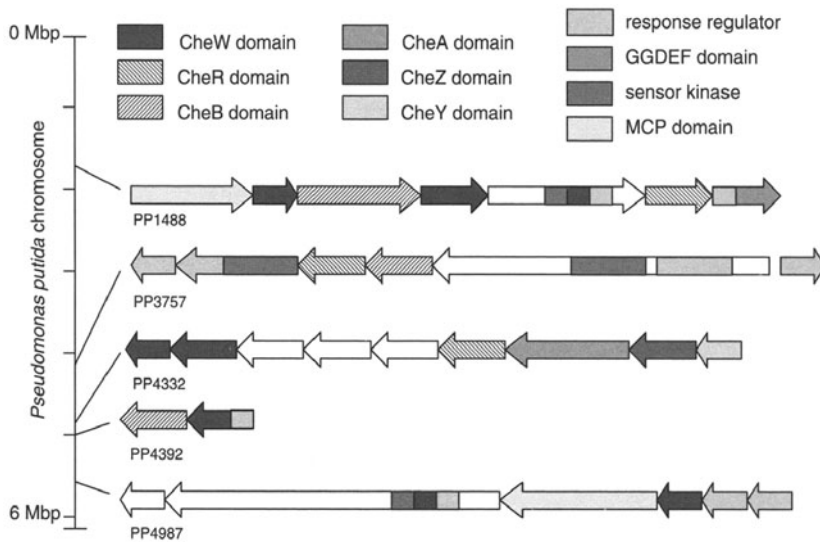


Figure 5. Position and organization of the major chemotaxis operons of *P. putida* KT2440.

7. TRAITS RELATED TO CELLULAR INTERACTIONS

Important interactions of bacteria include adhesion to animate and inanimate surfaces and biofilm formation, the production of stimulatory and inhibitory substances, signalling molecules and invasion factors, and defence mechanisms to hostile activities of other organisms.

7.1. Adhesins

Type IV pili mediate adhesion of bacteria to surfaces, including plants and fungi, and twitching motility¹⁶. The KT2440 chromosome contains two clusters of pilin genes between 715 and 741 kb (*fimT*, *pilE*, *pilACD*) and between 5682 and 5818 kb (*chpA*, *pilLJIHG* and *pilQNM*T), as well as *pilF* that is located at 986 kb. In contrast to other pseudomonads, like *P. stutzeri* and *P. fluorescens*, KT2440 lacks a copy of the nucleotide-binding pilus assembly protein gene, *pilB*, which is considered to be essential for pilus assembly^{40, 47}. Moreover, the genes for PilP, responsible for the multimerization of PilQ in the outer membrane⁴⁰, and PilO, responsible for the glycosylation of the pilin tip¹⁵, both of which are essential for the expression of pili¹⁷, are degenerated and collapsed into a single ORF (PP5081): The C-terminal half of *P. aeruginosa*

PilO protein exhibits 24% identity to the N-terminus of PP5081 and the 79 aa C-terminus of *P. aeruginosa* PilP protein has 37% identity to the C-terminus of PP5081. Extrapolating from the genome sequence to the phenotype, it seems likely that pilus formation may be severely affected in KT2440. The absence of *pilB* and the lack of pili in *P. putida* strain WCS358¹¹ would be consistent with this conclusion. On the other hand, a lack of PilB might potentially be compensated by its counterpart in the general secretion machinery, XcpR, and the degenerated PilOP fusion protein might still have a residual activity.

The *P. aeruginosa* genome contains three chaperone/usher pathway gene (*cup*) clusters. In Cup systems, pilin subunits are exported to the periplasm by the Sec-dependent pathway, whereas the usher forms a pore in the outer membrane to translocate the pilin to the cell surface to form fimbrial adhesins. *P. putida* has two loci of the chaperone/usher pathway. One locus (PP2363-PP2357) has the same organization as the *csu*-locus of *Vibrio parahaemolyticus* and two loci in the *P. aeruginosa* and *S. meliloti* genomes. The other locus (PP1888-PP1891) is homologous to the determinants of the *P. aeruginosa* CupB1 adhesin and to the CupC2 and CupC3 components of the secretory system.

The genome of *P. putida* encodes two unusually large proteins, PP0806 with a size of 6310 aa and PP0168 with a size of 8682 aa, the second largest bacterial protein described so far. On the basis of the study of Espinosa-Urgel *et al.*²⁰, who found that transposon insertion mutants of both proteins fail to adhere to the surface of plant seeds, both proteins, as well as another large protein (PP1449, 1508 aa), have been annotated as surface adhesion proteins. All three proteins exhibit a complex repetitive structure, an atypical amino acid composition, are threonine-rich and free of cysteine (Figure 6).

PP0168 contains three classes of peptide sequence repeats: Nine highly conserved N-terminal repeats of 100 aa, 29 highly conserved repeats with a size of 219 aa that form two subgroups (1–7, 8–29), and 5 haemolysin-type calcium binding repeats at the C-terminus. Moreover, a C-terminal domain shows similarity to a von Willebrand domain that is involved in adhesion and signal transduction⁴. The second largest protein, PP0806, consists of about 65 repeats with an average size of 85 aa. The first 35 repeats (with the exception of repeats 1 and 2) form 7 clusters of 5 repeats. Towards the C-terminal end, the repeats exhibit an increasing degree of sequence variation. A similar repeat structure was found for adhesion proteins of *Paeruginosa* (PA1874), *Salmonella* spp., *Enterococcus faecalis* and a surface protein of *Staphylococcus aureus* that is involved in biofilm development⁹. The C-terminus of PP0806 (600 aa) is similar to parts of RTX-family toxins. The third adhesin PP1449 contains an N-terminal haemagglutination activity domain and 9 haemagglutinin repeat domains.

The mucoid morphotype caused by hyperproduction of alginate is one hallmark of *P. aeruginosa* isolates obtained from chronically-infected lungs of

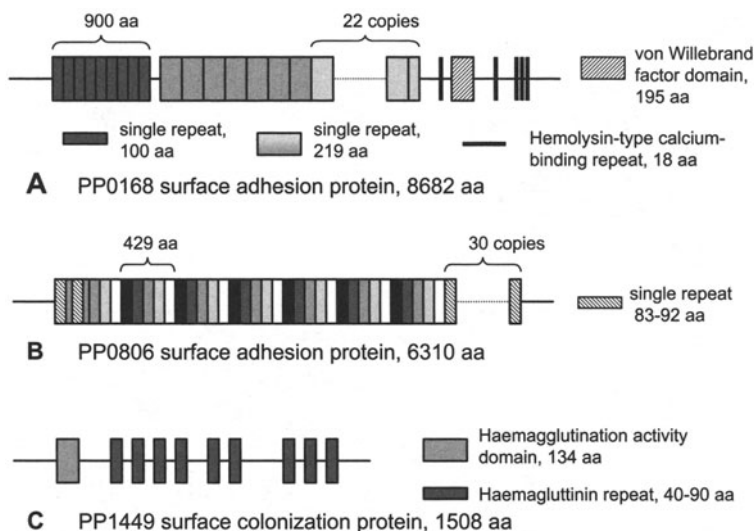


Figure 6. Domain organization and repeat structure of the three large adhesion proteins of *P. putida* KT2440.

cystic fibrosis patients³⁴. Evidence exists that alginate may be produced by *P. putida* strains upon antibiotic stress²⁶, but alginate biosynthesis by *P. putida* has not been investigated in detail. Of the 24 genes of the alginate regulatory and biosynthesis systems 23 are present in the KT2440 genome, though the determinant of the transcriptional regulator AlgM/MucC⁴⁸ is absent.

7.2. Damage of Eukaryotic Cells

P. putida is a saprophyte and considered to be non-pathogenic for plants and animals. Key determinants of virulence and virulence-associated traits present in the *P. aeruginosa* genome, but absent from that of the KT2440 genome include genes for exotoxin A, elastase, exolipase, phospholipase C, alkaline protease, a Type III secretion pathway, two Nramp manganese/iron transporters, and an operon for the synthesis of rhamnolipids. Quorum sensing, cell:cell signalling by quinolones and homoserine lactones, is a virulence-associated trait. The KT2440 genome does not contain homologs of *qsc*, *lasRI* and *rhlRI*, and correspondingly cells do not produce detectable amounts of homoserine lactones (L. Eberl, personal communication). Comparison of the KT2440 genome with those of the phytopathogens *Pseudomonas syringae*, *Ralstonia solanacearum*, *Xanthomonas campestris* and *Xylella fastidiosa* reveals that KT2440 lacks the genes of practically all known plant-related

virulence traits, such as type III secretion systems and corresponding secreted substrates^{22, 29, 57}, as well as plant cell wall degrading enzymes⁶. KT2440 thus lacks the key virulence determinants of pathogenic *Pseudomonads*. Genetic determinants that are shared between KT2440 and pathogenic species suggest that certain properties, such as adhesion and polymer biosynthesis, type IV pili, adhesins, stress-related proteins and master regulators such as GacA, which are commonly considered to be important for pathogenesis in both plants and animals⁶, may in fact simply be important for effective colonization and survival on surfaces, that is may be general survival functions, and not obligatorily related to pathogenesis, which involves damage of a host.

7.3. Antagonists

Bacteria produce a variety of antagonists, including low molecular weight antibiotics and high molecular weight polypeptide bacteriocins. Whereas low molecular weight antibiotics tend to be active against a broad spectrum of organisms, bacteriocins are generally active only against a narrow spectrum of closely related species.

P. aeruginosa produces three different types of chromosomally encoded bacteriocins: R-, F- and S-type pyocins. *P. aeruginosa* clones can be differentiated by their differential repertoire of pyocins, and pyocin typing is a robust and validated phenotypic method for strain typing in *P. aeruginosa*. R-type pyocins resemble inflexible and contractile tails of bacteriophages and kill sensitive bacteria by depolarization of the cytoplasmic membrane and inhibition of active transport. KT2440 encodes R2-type pyocin genes (PP3031-PP3066), the first 24 of which are syntenic with PAO1. S-type pyocins are soluble proteins and sensitive to proteases. Two S2-type pyocin genes (PP1305, PP1306) encode a protein with DNase activity, and an immunity protein that suppresses the activity of the pyocin, respectively. Both R and S pyocins are conserved in other pseudomonads. Other genes that might encode the production of bacteriocins are an ortholog of *cvpA*, which is essential for bacteriocin production in *E. coli*, and a putative bacteriocin cluster containing determinants of a putative bacterocin (peptide) synthase (PP2563), a hemolysin-type calcium-binding protein (PP2561) and its type I secretion system (PP2558- 2560).

Genes PP3779-3790 comprise a potential biosynthesis operon for an antibiotic peptide. The key genes encode an aminotransferase, a non-ribosomal peptide synthase, a diaminopimelate epimerase (paralog of the *dapF* gene) and a putative antibiotic efflux transporter. PP3783 that was annotated as a conserved hypothetical protein, belongs to a protein family (TIGR01762) which is involved in the chlorination of amino acid methyl groups and in the biosynthesis of antibiotics such as barbamide, syringomycin and the phytotoxin coronatine. Another operon for the biosynthesis of a secondary metabolite comprises

several genes for the biosynthesis of a polyketide (PP2777-PP2788). Whether or not the resulting molecule acts as a bacteriocin, siderophore or serves another function such as a signalling molecule, requires investigation.

8. CONCLUDING REMARKS

Genomic insights into the ubiquity and metabolic versatility of P. putida. The genome of KT2440 exhibits combinations of features characteristic of terrestrial, rhizosphere and aquatic bacteria, which thrive in either copiotrophic or oligotrophic habitats, and reveals that *P. putida* has evolved and acquired functions that equip it to thrive in diverse, often inhospitable environments, either free-living, or in close association with plants. The high diversity of protein families encoded by its genome, the large number and variety of small paralogous families, insertion elements, REP sequences, as well as the mosaic structure of the genome (with many regions of 'atypical' composition) and the multiplicity of mobile elements, reflect a high functional diversity in *P. putida* and are indicative of its evolutionary trajectory and adaptation to the diverse habitats in which it thrives. The unusual wealth of determinants for high affinity nutrient acquisition systems, mono- and di-oxygenases, oxido-reductases, ferredoxins and cytochromes, dehydrogenases, sulphur metabolism proteins, etc., for efflux pumps and glutathione-S-transferases ordinarily associated with protection against toxic substrates and metabolites, and for the extensive array of ECF sigma factors, regulators, and stress response systems, constitutes the genomic basis for the exceptional nutritional versatility and opportunism of *P. putida*, its ubiquity in diverse soil, rhizosphere and aquatic systems, and its renowned tolerance of natural and anthropogenic stresses. This metabolic diversity is also the basis of the impressive evolutionary potential of KT2440, and its utility for the experimental design of novel pathways for the catabolism of organic, particularly aromatic, pollutants, and its potential for bioremediation of soils contaminated with such compounds.

Genomic basis of the biosafety characteristics of KT2440. Genomic comparisons between the saprophytic KT2440 strain and plant- and animal-pathogenic *Pseudomonads*, particularly *P. aeruginosa* and *P. syringae*, have defined the characteristics that account for the different lifestyles and biological properties of these organisms. KT2440 lacks the determinants of key virulence factors of pathogenic *Pseudomonads* that mediate host damage, including exotoxins, specific hydrolytic enzymes, type III secretion systems, factors mediating hypersensitive responses, etc. This genomic analysis has thus provided a definitive basis of the biosafety characteristics of *P. putida* strain KT2440, and generated a database upon which the environmental and biotechnological behaviour of this strain can be interpreted.

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THE GENOME OF *PSEUDOMONAS SYRINGAE* TOMATO DC3000 AND FUNCTIONAL GENOMIC STUDIES TO BETTER UNDERSTAND PLANT PATHOGENESIS

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1. INTRODUCTION

Pseudomonas syringae is a member of the gamma subgroup of the Proteobacteria and a plant pathogen that infects many different plants³⁰. Certain *P. syringae* strains can infect only one or a few plant species, and on this basis, *P. syringae* has been separated into greater than 50 pathovars¹⁰⁶. While *P. syringae* will eventually kill plant cells (i.e., it is necrogenic), it is typically considered a biotrophic pathogen and it can live on plant leaves as an epiphyte^{3, 49}. The host specificity that *P. syringae* displays is at least partly due to pathogen avirulence genes that encode proteins (i.e., Avr proteins) that trigger the disease resistance (*R*)-gene-based plant innate immune system in resistant plants⁵³. One of the plant defense responses triggered by Avr proteins is the hypersensitive response (HR), which is a programmed cell death of plant tissue that is associated with successful defense against pathogens. *P. syringae* mutants unable to elicit an HR led to the discovery of *hrp* genes—the genes

that encode the *P. syringae* type III proteins secretion system (TTSS)⁶³. *P. syringae* pathogenesis relies on the TTSS and the effector proteins it translocates into host cells similar to most of the animal and plant pathogens that possess TTSSs²⁷.

P. syringae tomato DC3000 has become a model *P. syringae* strain to study molecular plant pathology because it infects an important agricultural crop, tomato and *Arabidopsis thaliana*, a genetically amenable model plant¹⁰¹. The complete genome sequence of *Arabidopsis* has been determined and, recently, The Institute for Genomic Research (TIGR) has finished sequencing and annotating the DC3000 genome²³. In this review, we first highlight some of the general features of the DC3000 genome and information on *P. syringae* virulence factors revealed by the Buell *et al.* report²³. Later portions of this chapter describe several functional genomic studies using the DC3000 genome while the genome was still in the draft stages that revealed much about the TTSS of DC3000. For additional information about the DC3000 genome we refer the reader to Buell *et al.*²³, the TIGR Comprehensive Microbial Resource webpage (<http://www.tigr.org>) and the *P. syringae* webpage (<http://pseudomonas-syringae.org/>).

2. THE GENERAL FEATURES OF THE DC3000 GENOME

The general features of the DC3000 genome are summarized in Table 1. The DC3000 genome is 6.5 Mb in size consisting of one circular chromosome (6,397,126 bp) and two plasmids, pDC3000A (73,661 bp) and pDC3000B (67,473 bp)²³. The DC3000 genome is the largest pseudomonad and bacterial plant pathogen genome published to date^{31, 43, 76, 87, 94, 97, 102}, although, the

Table 1. General features of the DC3000 genome²³.

Feature	
Total genome size	6.5 Mb
Chromosome size	6,397,126 bp
Plasmid pDC3000A	73,661 bp
Plasmid pDC3000B	67,473 bp
G+C content	58.34%
Mobile genetic elements	7% of genome
Predicted ORFs	5,763
Percent coding	86.48%
No. of ORFs with putative roles	3,510
No. of virulence-related ORFs	298

predicted size of the draft sequence of *P. fluorescens* indicates that it will be a bit larger than DC3000. The G+C percent for the DC3000 chromosome, pDC3000A and pDC3000B are 58.4, 55.1 and 56.1, respectively. There are 5,763 identified ORFs in the DC3000 genome of which about 61 percent have putative functions. A complete breakdown of the different role categories can be found in Buell *et al.*²³.

Because *P. syringae* grows in the apoplast, the intercellular spaces of plant leaves and a relatively undefined niche in terms of available nutrients, it was of special interest to try to find clues based on the genome that may identify important nutrient sources in the apoplast. As noted in Buell *et al.*²³, the DC3000 genome contains three copies of gamma-aminobutyric acid (GABA) transaminase, whereas *P. aeruginosa* PAO1 has only one copy of this gene. This is interesting because the apoplast has been reported to have millimolar concentrations of GABA during infection of tomato with a biotrophic fungus⁹⁵. The ability to take up compounds is also a critical factor in determining whether a compound is being used as a nutrient. DC3000 possesses many different proteins involved in transport similar to other sequenced pseudomonads. However, the DC3000 genome appears to have a greater ability to transport sugars. For example, there are 15 ABC transporters encoded by the DC3000 genome and among these some are predicted to transport arabinose, xylose, ribose and other plant produced sugars, while *P. putida* and *P. aeruginosa* appear to encode only four ABC transporters specific for sugars²³. In contrast, *P. putida* and *P. aeruginosa* appear better equipped at transporting amino acids than DC3000 because their genomes contain genes that encode 21 APC transporters, while the DC3000 genome only appears to encode four^{23, 52}. These observations provide new directions for researchers to explore the nuances of *P. syringae* pathogenesis.

At the time of writing this chapter, the only published pseudomonad genome sequences were from DC3000²³, the opportunistic animal pathogen *P. aeruginosa* PAO1⁹⁷ and the soil saprophyte *P. putida* KT2440⁷⁶. However, the draft sequence of *P. s. syringae* B728a, a *P. syringae* strain that is a good epiphyte, is available from The Joint Genome Institute (JGI) (www.jgi.doe.gov), and the genome of *P. syringae phaseolicola* 1448A, a well-studied bean pathogen, is currently being sequenced by TIGR (www.tigr.org). Moreover, the draft genomes of three *P. fluorescens* strains are available; strain Pf0-1 is available from JGI, Pf-5 from TIGR and SBW25 from The Sanger Institute (www.sanger.ac.uk). Comparing the genome of DC3000 to these other pseudomonad genomes will be extremely useful for the identification of DC3000 genes that may contribute to plant pathogenicity. An example, of how these comparisons could give insights into *P. syringae* biology is illustrated above for sugar transporters. When the two other finished pseudomonad genomes, *P. aeruginosa* PAO1 and *P. putida* KT2440, were compared to the

5,608 ORFs in DC3000, 4,449 were found to have homologs ($E < 10^{-5}$) in either *P. aeruginosa* or *P. putida*, 3,797 were present in all three genomes and 1,159 were found to be unique to DC3000²³. Many of these unique genes may contribute to lifestyles of *P. syringae* in unique environments, including pathogenesis, and, therefore, warrant further study.

3. THE GENOMICS OF VIRULENCE

Below we cover specific virulence factors identified in the DC3000 genome and how these may contribute to *P. syringae* pathogenesis. Table 2 shows a summary of the known virulence factors found in the genome of DC3000²³. Additional information about DC3000 virulence factors is available in Buell *et al.*²³, other review articles¹⁴, and in this book series, which includes several chapters on *P. syringae* virulence factors.

3.1. Known *P. syringae* Virulence Factors Encoded by the DC3000 Genome

A functional TTSS is required for *P. syringae* pathogenesis⁴. The TTSS system of *P. syringae* is encoded by *hrp* (hypersensitive response and pathogenicity) and *hrc* (*hrp* conserved) genes. The promoters of *hrp/hrc* genes contain a *cis* element, called the Hrp box, which is recognized by the alternative sigma factor HrpL¹⁰³. The *hrp/hrc* genes are all clustered together in the central portion of the Hrp pathogenicity island (Pai)². Substrates of the TTSS

Table 2. Known and potential virulence genes found in DC3000²³.

Function	No. of genes
TTSS	34
TTSS substrates (and candidates)	40 ¹⁷
ABC importers	15
Type I secretion system (ABC exporters)	5
Type II secretion system	11
Siderophores	14
Phytotoxins and phytohormones	26
Adhesions	26
Type IV pili	23
Extracellular polysaccharides	22
Flagella	34
Cell wall-degrading enzymes	4
ROS resistance	22

fall into two classes: Helper proteins, which assist in the delivery of proteins into plant cells, or effector proteins, which are delivered or translocated into host cells. Since members of both classes are secreted, they have been named Hops for *Hrp* outer proteins. A subset of effectors were identified as avirulence (Avr) proteins because these proteins, when expressed heterologously in a virulent pathogen, render the pathogen nonpathogenic^{32,61}. It is hypothesized that *hrp/hrc* genes were acquired horizontally. If so, then for the TTSS system to be useful to (and selected for) a *P. syringae* ancestor, genes encoding substrates would also need to be carried within the *Hrp* *Pai*. Indeed, both regions flanking the *hrp/hrc* genes carry effector genes. One of these regions is called the conserved effector locus (CEL) and this region, as its name implies, is conserved in all *P. syringae* pathovars tested^{2,33}. Several of the effectors encoded in this region contribute significantly to virulence based on strong virulence phenotypes^{2,11}, whereas most other effectors have weak virulence phenotypes suggesting that they may be functionally redundant. The other region is variable between even closely related *P. syringae* strains and was named the exchangeable effector locus (EEL)². The EEL contains different effector genes in different *P. syringae* strains and it has been recently mined for novel effector genes³³.

The genes encoding the DC3000 TTSS apparatus were identified prior to the availability of the DC3000 genome sequence. Thus, the primary goal once the sequence information was obtained was to identify additional DC3000 effectors. Because effector mutants generally have weak virulence phenotypes, effector genes away from the *Hrp* *Pai* were difficult to isolate using conventional genetic approaches. Thus, the DC3000 genome sequence greatly facilitated the identification of effector genes. Functional genomic studies using the DC3000 genome summarized in Figure 1 and described below have led to the identification of many Hops^{26,38,44,45,81,108}. The current inventory of Hop proteins and candidate Hops is shown in Table 3. The list now contains 7 helper proteins and 33 effector proteins based on demonstrated secretion, translocation or the presence of a homolog in another *P. syringae* pathovar. There are 17 additional candidate Hops that await testing. Therefore, the Hop inventory is likely to be greater than 50 and it is larger already than the Hop inventories found in other TTSS-containing microorganisms. An up-to-date Hop inventory list could be found on the *P. syringae* website (<http://pseudomonas-syringae.org>). Whether the large *P. syringae* Hop inventory is because *P. syringae* has more TTSS substrates than other TTSS-containing microbes or the *P. syringae* researchers have searched more aggressively for additional TTSS substrates is an open question that should be answered in the future.

Several *P. syringae* pathovars, including DC3000 make phytotoxins and phytohormones⁷⁵. One such phytotoxin is coronatine (COR), which is a small diffusible molecule responsible for the formation of chlorotic halos

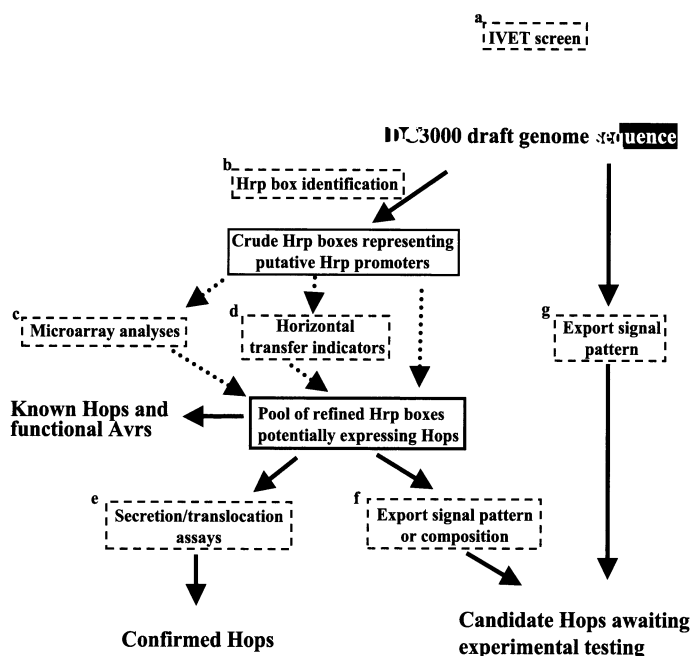


Figure 1. Strategies used to identify DC3000 TTSS Hops. The flowchart outlines the different strategies that have been used to identify DC3000 TTSS-regulated genes and Hops. (a) IVET screens in conjunction with the DC3000 genome information allowed for identification of genes that were induced *in planta* that also possessed Hrp promoters¹⁷. (b) Putative Hrp boxes were identified in the DC3000 genome by using hidden Markov model and string searches^{38, 108}. (c) ORFs that contained putative Hrp promoters upstream were tested directly with microarray and/or Northern analyses to determine if they were expressed in a HrpL-dependent manner^{38, 108}. (d) Because most known *P. syringae* *hop/avr* genes have indications of being horizontally transferred⁵⁴, determining whether a candidate *hop* gene possessed indications of being horizontally acquired enriched for *hop* genes⁸¹. (e) Candidate *hop* genes were tested experimentally for the type III-dependent secretion/translocation to determine whether they encoded Hop proteins^{45, 81}. (f) Further bioinformatic analyses were done on candidate *hop* genes to determine whether they encoded proteins with biochemical properties or export signal patterns consistent with the protein being a Hop^{45, 81}. (g) Computer algorithms were used to search the DC3000 genome independent of Hrp promoters for ORFs predicted to encode proteins that shared the biochemical properties and export signal patterns contained on Hop and Avr proteins⁸¹.

surrounding the necrotic lesions caused by *P. syringae* pathovars that make it⁷⁵. The structure of COR consists of two distinct components, coronafacic acid (CFA) and coronamic acid (CMA), which are joined by an amide linkage¹⁴. CFA is structurally related to methyl jasmonate (MeJA), a plant hormone derived from the octadecanoid pathway⁸³. CMA exhibits structural similarities to 1-aminocyclopropane-1-carboxylate, the precursor of ethylene. Both JA and

Table 3. Confirmed and predicted DC3000 TTSS substrates.

DC3000 Hops ^a	TIGR ORF Call ^b	Also known as ^c	Evidence for transcript ^d	Sec. and/or transloc. ^e	References
Helpers					
HopPmaG _{Pto}	PSPTO0852	ORF21	ND	ND	[45], [82]
HopPmaH _{Pto}	PSPTO4101	ORF12, HCO31	Yes	ND	[45], [82]
HopPtoP	PSPTO2678	HrpW-related, Chp5	Yes	T	[45], [82]
HrpA _{Pto}	PSPTO1381	ipx4	Yes	S	[87]
HrpK _{Pto}	PSPTO1405	ipx5, 6	Yes	S ^h	[2]
HrpW _{Pto}	PSPTO1373	HCO37, ipx37	Yes	S	[2]
HrpZ _{Pto}	PSPTO1382	NA	ND	S	[2]
Effectors					
AvrE _{Pto}	PSPTO1377	NA	Yes	ND	[2]
AvrPphE _{Pto}	PSPTOA0012	NA	Yes	T	[38]
AvrPpiB1 _{Pto}	PSPTO1022	HCO11, ipx8 ^g	Yes	ND	[38]
AvrPpiB2 _{Pto}	PSPTOA0005	HCO11, ipx8 ^g	Yes	ND	[38]
AvrPtoDC3000	PSPTO4001	NA	Yes	S	[100]
AvrPtoB	PSPTO3087	VirPphA _{Pto} , HCO10, ipx7	Yes	S	[58]
HopPmaI _{Pto}	PSPTO4776	ORF10, Chp2	Yes	ND	[45], [82]
HopPmaJ _{Pto}	PSPTO1179	NA	ND	ND	[45]
HopPsyA _{Pto}	PSPTO5354	ipx32	Yes	ND	[38]
HopPtoA1	PSPTO1372	CEL ORF5	Yes	S	[10]
HopPtoA2	PSPTO4718	NA	No	S	[10]
HopPtoB1	PSPTO1406	EEL ORF1	ND	S ^h	[2]
HopPtoC	PSPTO0589	AvrPpiC2 _{Pto} , HCO12	Yes	S, T	[82], [93]
HopPtoD1	PSPTO0876	AvrPphD1 _{Pto} , HCO13	Yes	S	[82]
HopPtoD2	PSPTO4722	AvrPphD2 _{Pto}	Yes	S	[21], [34], [82]
HopPtoE	PSPTO4331	ORF3, Chp1, HolPtoV	Yes	S, T	[82], [109]
HopPtoF	PSPTO0502	HolPtoAD, AvrPphF ORF2 _{Pto}	Yes	S ⁱ	[38]

Table 3. Continued.

DC3000 Hops ^a	TIGR ORF Call ^b	Also known as ^c	Evidence for transcript ^d	Sec. and/or transloc. ^e	References
HopPtoG	PSPTO4727	ORF4, HopPtoW, HCO27	ND	S, T	[82]
HopPtoH	PSPTO0588	ORF2, HCO50	ND	S	[82]
HopPtoI	PSPTO4691	HolPtoS, ORF1	ND	S	[82]
HopPtoJ	PSPTO1568	AvrXv3 _{Pto}	No	S	[82]
HopPtoK	PSPTO0044	AvrRps4 _{Pto} , HolPtoAB	No	T	[82]
HopPtoL	PSPTO2872	ORF29	ND	S	[82]
HopPtoM	PSPTO1375	CEL ORF3, HCO12, 13	Yes	S	[2], [11]
HopPtoN	PSPTO1370	CEL ORF7, HCO14	Yes	S ^j	[38], [93]
HopPtoQ	PSPTO0877	ORF19, HolPtoQ	ND	T ^j	[45]
HopPtoS1	PSPTOA0018	ORF5, HopPtoO	Yes	S, T	[45], [82]
HopPtoS2	PSPTO0501	ORF30	ND	S	[82]
HopPtoS3	PSPTO4594	ORF31	ND	S ^h	[82]
HopPtoT1	PSPTOA0019	ORF16	ND	T ^h	[82]
HopPtoT2	PSPTO4593	HolPtoU2	ND	S ^h	[45]
HopPtoV	PSPTO4720	ORF17	ND	S ^h , T ^j	[23]
HopPtoW	PSPTO0901	HolPsyAG _{Pto} , ORF28	ND	T ^j	[44]
Candidate Hops					
HolPsyAH _{Pto}	PSPTO0905	NA	ND	ND	[23], [44]
HolPsyAI _{Pto}	PSPTO0906	NA	ND	ND	[23]
HolPtoAA	PSPTO4703	NA	ND	ND	[45]
HolPtoQ2	PSPTO4732	HolPtoQ-like	ND	ND	[23]
HolPtoR	PSPTO0883	ORF61	Yes	ND	[45]
HolPtoY	PSPTO0061	ORF11, HrpA-like	ND	ND	[38]
HolPtoZ	PSPTO4597	ORF26, ipx69	No	ND	[45], [82]

HolPtoZ2	PSPTO4588	ORF15	ND	ND	[82]
HopPtoB2	PSPTO4993, 4996	HolPtoAC	No	ND	[38], [45]
HopPtoD1-related	PSPTO4724, 4726	NA	ND	ND	[23]
HopPtoS1-related	PSPTO4592	HolPtoO-related	ND	ND	[23]
HopPtoT1-related	PSPTO4590	HolPtoU-related	ND	ND	[23]
ipx47	PSPTO0875	NA	Yes	ND	[23]
ipx53	PSPTO5061	NA	Yes	ND	[16]
ORF1152	PSPTO0474	NA	Yes	ND	[23]
ORF20	PSPTO0869	NA	ND	ND	[82]
ORF36	PSPTO4188	NA	ND	ND	[23], [82]

^aHop names reflect the current names for each and represents an updated form of the table shown in Collmer *et al.*²⁶.
^bThese are the ORF names given to each ORF by TIGR and are the names attached to each ORF in GenBank.
^cWe have attempted to give the alternate names that were given to Hops in publications that used the DC3000 draft genome. Names with the ipx prefix are from Boch *et al.*¹⁷, ORF names are from Petnicki-Ocwieja *et al.*⁸¹, names with the Hol prefix are from Guttman *et al.*⁴⁵, and Chp and HCO names are from Zwiesler-Vollrick *et al.*¹⁰⁸. NA (i.e., not applicable) indicates that we know of no alternate names.
^dConfirmation of transcription were based on microarrays or RNA gel blots reported in either Zwiesler-Vollrick *et al.*¹⁰⁸ or Fouts *et al.*³⁸.
^eStatus of DC3000 TTSS substrate testing is indicated by S (secretion shown), T (translocation shown), or ND (not done).
^fHopPmaG_{Pto} was reported to be translocated into plant cells⁴⁵ although its sequence suggests a function within the bacterial envelope.
^gThe IVET and HCO screen identified an *avrPpiB1* allele^{75, 108}, but it is not possible to determine which one of the two alleles was identified.
^hUnpublished results from the Alfano Laboratory.
ⁱUnpublished results from the Tang Laboratory.
^jPersonal communication with A. Collmer.

ethylene are important plant defense hormones⁷⁷. COR and MeJA induce analogous biological responses in *Arabidopsis* seedlings, and the COR-insensitive *Arabidopsis* mutant plants are insensitive to MeJA and resistant to *P. syringae* infections³⁷. This led to the hypothesis that COR functions as a molecular mimic of MeJA and promotes colonization of host tissue by altering plant defense signaling pathways⁵⁹. The synthesis of CFA and CMA in DC3000 is catalyzed by two clusters of chromosomal genes separated by 26 kb²³. This is different from many other *P. syringae* pathovars in which the two gene clusters are tightly linked and plasmid-encoded¹⁴. There is no evidence in the DC3000 genome for the biosynthesis of syringomycin and related lipodepsinonapeptide phytotoxins. However, DC3000 does contain five nonribosomal peptide synthetases in three separate gene clusters (PSPTO2829, 2830, 4518, 4519, 4699) that may direct the biosynthesis of novel peptide or lipodepsipeptide phytotoxins. The function of these genes in bacterial virulence remains to be determined.

Production of the phytohormone indole-3-acetic acid (IAA) has been detected in a large number of *P. syringae* pathovars where it is synthesized from L-tryptophan⁴¹. There are three genes related to IAA production known to be present in many *P. syringae* pathovars⁷⁰. *iaaM* encodes a tryptophan monooxygenase, and *iaaH* encodes an indoleacetamide hydrolase. The third gene, *iaaL*, encodes IAA-lysine ligase, which is not involved in IAA biosynthesis *per se*, but instead, converts IAA to a less active derivative by conjugating it to lysine. Typically, the *iaaM* and *iaaH* genes of *P. s. syringae* are clustered in a chromosomal region distinct from *iaaL*. DC3000 harbors *iaaM* (PSPTO0518) *iaaH* (PSPTO4204) and *iaaL* genes (PSPTO0371) each in different regions of the chromosome²³. Interestingly, the promoter of *iaaL* has a functional Hrp box motif³⁸, suggesting a role of IAA in *P. syringae* virulence. If IAA-lysine ligase does indeed reduce the activity of IAA, because production of this enzyme appears to be HrpL-dependent, it is tempting to speculate that the *P. syringae* TTSS and IAA function at different stages of plant pathogenesis.

Extracellular polysaccharides (EPS) are thought to enhance bacterial survival by generating a hydrogenated barrier that minimizes direct contact with the plant defense compounds. In addition, EPS may protect bacterial cells from desiccation, concentrate minerals and nutrients and improve attachment to plant surfaces during epiphytic growth. Phytopathogenic pseudomonads are known to produce two types of EPSs, alginate and levan^{36, 62}. Alginate is a copolymer of β -D-mannuronic acid and α -L-guluronic acid, linked together by 1–4 linkages. The production of alginate has been linked to the epiphytic fitness and the production of water-soaked lesions in pathogenic *Pseudomonas* bacteria¹⁰⁷. In *P. aeruginosa*, the production of alginate involves at least 24 genes organized in three major clusters of the bacterial chromosome³⁹.

With the exception of *algC*²⁹, the rest of the genes that catalyze the synthesis of alginate are clustered in the same locus of the chromosome. These genes are organized almost identically in the DC3000 genome²³. Levan, another EPS, is a polymer of β -(2,6)-polyfructan with extensive branching through β -(1,2)-linkages^{6, 39}. The formation of both linkages is catalyzed by levansucrase. Three highly conserved genes encoding levansucrases are present in DC3000 (PSPTO1453, 2305, 0032)²³, corresponding to the *lscA*, *lscB* and *lscC* genes in the *P. syringae* glycinia PG4180 strain⁶². Like their cognate genes in PG4180 strain, two of the levansucrase genes (PSPTO1453, 2305) have a chromosomal location while the third gene (PSPTO0032) resides on a plasmid.

3.2. Other Potential Virulence Factors Identified in the DC3000 Genome

The pseudomonads *P. aeruginosa* and *P. fluorescens* have been shown to possess genes in their genomes for all of the protein secretion systems known to exist in Gram-negative bacteria except for the type IV protein secretion system⁶⁷. Although an extensive analysis has yet to be performed on the DC3000 genome, it is likely to show similar findings. DC3000 does have several ORFs that are similar to components of type I secretion systems and appears to have a functional type II protein secretion system²³. Both type I and type II secretion systems are often virulence factors^{16, 88, 89}. However, it is unclear what substrates are traveling both of these protein secretion systems in DC3000. Because proteins that travel type II pathways are often degradative enzymes, Buell *et al.*²³ identified several potential type II substrates, which included a pectin lyase (PSPTO4283), an endo-polygalacturonase (PSPTO3960) and three enzymes predicting cellulolytic activity (PSPTO1029, 3534, 0905). Pectic enzymes and cellulases were previously identified in other *Pseudomonas* spp., and pectate lyase has been shown to contribute to disease symptoms produced by *P. syringae* pv. lachrymans¹².

Fluorescent pseudomonads are known to produce a number of siderophores, most commonly the siderophore pyoverdinin^{24, 28}. Siderophores are low molecular weight compounds that act as iron(III) chelators and are important virulence factors because of the low iron availability in plants. These compounds are generally peptides made by nonribosomal peptide synthetases (NRPS) and bind iron outside of the bacterium. Ferrisiderophores are brought back into the bacterial cell via specific receptors that function in concert with the TonB protein, which supplies the energy needed for uptake²⁰. Clusters of genes similar to those required to make pyoverdinin and pyochelin have been identified in the DC3000 genome²³. Moreover, there is also an ORF (PSPTO2602) predicted to encode an NRPS that produces the yersiniabactin

siderophore from *Yersinia* spp.^{23, 72}. Along with siderophores, another important consideration is whether there are many genes encoding siderophore receptors. Pseudomonads are known to have many different TonB-dependent siderophore receptors. There are 35 siderophore receptors identified in *P. aeruginosa*⁹⁷ and the DC3000 genome contains homologs to 23 of these²⁸. The propensity of genes that encode enzymes that make siderophores or encode siderophore receptors highlights the emphasis that pseudomonads put towards iron acquisition.

The ability of bacterial pathogens to attach to plant cells has not received nearly as much attention by researchers as attachment to animal cells by animal pathogens. However, there are several indications that many of the same strategies used by animal pathogens are used by plant pathogens to adhere to plants. For example, type IV pili (Tfp)¹⁰⁰ and fimbriae are made by *P. syringae*^{84, 86}. Tfps have been shown to promote the epiphytic fitness of *P. syringae*⁴⁹. DC3000 possesses the genes in its genome to encode a complete Tfp biogenesis system²³. DC3000 also carries three homologs (PSPTO3210, 3229, 3214) of the *Bordetella pertussis* afimbral adhesion filamentous hemagglutinin (FHA)^{23, 66}. FHA has been found in several other plant pathogens including *Erwinia chrysanthemi*, *Erwinia carotovora*, *Ralstonia solanacearum*, *Xylella fastidiosa* and *Xanthomons campestris* *campestris* and *Xanthomons campestris* *citri*^{13, 23, 55, 87}. Interestingly, there are 14 probable FHA homologs in *R. solanacearum* suggesting that they are important virulence factors⁸⁷. The FHA homolog, HecA, in *E. chrysanthemi* has been shown to contribute to virulence confirming that these proteins are important for bacterial plant pathogenicity⁸⁵. The availability of the DC3000 genome will facilitate experiments to decipher the mechanisms that bacterial pathogens use to attach to plant cells.

The triggering of the plant innate immune system often results in the production of reactive oxygen species (ROS)^{74, 79}. ROS act as signaling molecules in processes such as programmed cell death, pathogen defense, and abiotic stress responses. Because ROS are highly reactive and damaging to cellular components it is often toxic to microorganisms⁵⁸. A longstanding hypothesis for researchers studying bacterial plant pathogens is that bacterial enzymes that protect against ROS present in the apoplast are important virulence factors. The enzymes that can protect against ROS include catalase, superoxide dismutase (SOD) and peroxidases. SODs convert superoxide to H₂O₂, which is converted to water by catalase. Catalases and SODs are virulence factors in the plant pathogens *Agrobacterium tumefaciens* and *E. chrysanthemi*^{90, 105}. DC3000 has three catalase genes (PSPTO3582, 5263, 4530) and three SOD genes (PSPTO1338, 4363, 4459), all chromosomally located²³. Catalases have not been shown to be virulence factors in *P. syringae*, although it has been reported that *P. syringae* strains contain multiple catalases⁶⁰. Mutations in all three of the catalase genes of DC3000 should determine whether these enzymes act as

virulence factors. SOD was reported not to be a virulence factor in *P. s. syringae* B728a⁵⁶, but now that DC3000 is known to have multiple SODs, the lack of a virulence phenotype in the earlier report might be due to SOD redundancy. There are several other genes identified in the DC3000 genome that may encode enzymes that protect DC3000 from ROS²³.

4. FUNCTIONAL GENOMICS ON DC3000

Described below are several functional genomic approaches recently taken with DC3000, some of which have been previously reviewed^{5, 26, 44}. With the exception of the IVET screen, which can identify any gene induced in the host, most of these approaches were focused on learning more about the TTSS of DC3000. These approaches are summarized in Figure 1, which shows a flowchart of how each approach was used to identify TTSS substrates.

4.1. IVET Screen to Find Genes Induced *in planta*

Many DC3000 genes induced during infection of *Arabidopsis* were recently identified¹⁷. The researchers used the strategy of *in vivo* expression technology (IVET) pioneered by M. Daniels and colleagues studying *Xanthomonas campestris* interactions with plants⁷⁸ and later popularized by *Salmonella* researchers⁶⁹ and other researchers studying animal pathogens and other bacteria^{48, 82}. The basic strategy uses a conditional mutation in a gene that encodes a product required for growth. Bacterial growth of this mutant cannot occur unless the required gene is expressed in the mutant. For complementation, a promoter library is made such that each random DNA insert is upstream of the required gene. Thus, if the promoter is active *in vivo* in the mutant the required gene will be expressed and the mutant will be complemented regaining the ability to grow in the host. In the DC3000 IVET experiments the researchers used a DC3000 *hrcC* deletion mutant, which cannot replicate in plant tissue because *hrcC* encodes a required component of the TTSS. A promoter library was made upstream of *hrcC*. Thus, if a construct contained an active promoter, HrcC would be produced allowing for a functional TTSS to be assembled. The constructs also carried the *uidA* gene to discriminate between promoters that are constitutively producing GUS and forming blue colonies on rich media containing X-gluc from those genes that are induced *in planta* and remain white on X-gluc plates.

The researchers identified 268 unique clones from their promoter library that were able to complement the *hrcC* mutant *in planta* indicating that these promoters were expressed *in planta*¹⁷. These promoters were referred to as *ipx*

promoters for *in planta*-expressed. Flanking nucleotide sequence was obtained for 79 of the *in vivo* complemented *P. syringae* strains. Thirty-four inserts contained promoters of known virulence genes, 16 were promoters of known genes that were not previously associated with virulence, 14 were genes with unknown function and 15 were promoters upstream of unique genes with no homologs in the databases¹⁷. Not surprisingly, many of the induced genes that were known virulence genes were related to the TTSS. The IVET screen picked up five promoters from within the *hrp/hrc* cluster moiety of the Hrp Pai, which encodes the TTSS apparatus² and identified four promoters within the CEL of the Hrp Pai confirming that these genes have active Hrp promoters. It also identified several genes away from the Hrp Pai that encode helper or effector proteins. These include the effectors HopPtoA2¹⁰, AvrPphE_{Pto}⁸¹, AvrPpiB_{Pto}³⁸, HopPmaI_{Pto}⁴⁵, AvrPtoB⁵⁷ and HopPtoE⁸¹ and the helper protein HopPmaH_{Pto}⁴⁵, a pectate lyase-related protein that appears to share characteristics with HrpZ and other harpins⁴. *ipx* promoters were also identified upstream of other virulence genes such as several coronatine-related genes, a catalase gene, an alginate biosynthesis gene and a *syxE*-like nonribosomal peptide synthetase gene¹⁷.

The IVET screen also identified promoters upstream of known genes previously not known to be involved in pathogenicity or for growth in plants. Many of these promoters were associated with metabolism, which may provide clues to what enzymes DC3000 needs to flourish in the apoplast¹⁷. Among these genes were genes encoding a carboxypeptidase, a diaminopimelate decarboxylase and an acetolactate synthase. Other genes identified included genes involved in thiamine biosynthesis, an ABC transporter and a two-component regulatory system. Interestingly, both the thiamine biosynthesis and the carboxypeptidase promoters contained Hrp boxes indicating that they probably require the HrpL sigma factor and are expressed with the TTSS. Several promoters identified in the IVET search were either upstream of genes encoding products homologous to proteins of unknown function or not homologous to any proteins in the databases. While these promoters currently do not reveal much about DC3000 pathogenicity, they may be a virtual treasure trove as we begin to look more deeply at *P. syringae*-plant interactions.

4.2. Searching the Genome for Hrp Boxes to Identify TTSS Genes

P. syringae TTSS promoters contain a *cis* element, called the Hrp box. When the DC3000 draft genome became available it was searched genome-wide for Hrp boxes. The Hrp box is recognized by the alternative sigma factor HrpL, which is used in the transcription of most of the TTSS-related genes in *P. syringae*¹⁰³. The consensus sequence of the Hrp box was originally

considered to consist of a GGAACC 15/16 bp CCAC within a *hrp* or *avr* gene promoter^{51, 93, 104}. However, Fouts *et al.*³⁸ recognized that several known effector gene promoters contained atypical Hrp boxes. A hidden Markov model (HMM) was used to search typical and atypical Hrp boxes. This search yielded greater than 200 putative Hrp promoters in the genome of DC3000 with differing *E* values representing how similar each were to the consensus Hrp box³⁸. Another study identified putative Hrp boxes using a string search that encompassed several of the variations known to occur in DC3000 Hrp boxes and identified 73 putative Hrp boxes in the genome of DC3000¹⁰⁸. Microarray and RNA blot analyses showed that many of these genes were induced in Hrp-inducing conditions and dependent on either HrpL or HrpS, a regulatory protein that acts upstream of HrpL. These searches revealed which known effector genes from other bacterial plant pathogens were present in DC3000. Also, they showed that some Hrp promoters express genes unrelated to the TTSS and that some Hrp boxes of known effector genes are atypical indicating that other “real” TTSS-related genes await discovery within a relatively large pool of candidate genes identified using these approaches. These last two points highlighted the need to identify effectors based on characteristics other than their promoters.

4.3. Translational Reporter Fusions to Identify TTSS Effectors that are Translocated into Plant Cells

Two important assays have been used in *P. syringae* and other plant pathogens to identify translocated effectors and/or to determine whether a candidate effector is translocated into plant cells. Cleverly, Guttman *et al.*⁴⁵ designed a transposon such that it would make translational fusions between any ORF that the transposon recombined into and a truncated AvrRpt2 Avr protein. AvrRpt2 is recognized by *Arabidopsis* ecotypes containing the *RPS2* resistance gene^{15, 73}. Importantly, the transposon tool that these researchers used contained a truncated *avrRpt2*, which encodes an AvrRpt2 protein lacking its N-terminal secretion signals and is not translocated into plant cells unless it is fused in frame to another gene that provides type III secretion signals. Guttman *et al.*⁴⁵ used this AvrRtp2 transposon to identify 13 effectors from *P. syringae* maculicola ES4326⁴⁵. Moreover, this AvrRpt2 translocation assay has been used by several researchers to test directly whether a DC3000 candidate effector was translocated into plant cells^{11, 21, 45, 81, 108}.

The second translocation assay that has become important in determining whether a TTSS substrate is a translocated effector utilizes effector fusions with adenylate cyclase from *B. pertussis*. This assay was first used to show that *Yersinia enterocolitica* effectors were translocated into animal cells⁹⁶ and recently has been used in plant pathosystems²⁵. The assay is based on the

requirement of the calmodulin protein for adenylate cyclase to be active and produce cAMP from ATP. Because calmodulin is not present in bacteria, an active effector adenylate cyclase fusion indicates that the effector contains sufficient type III secretion signals to target the fusion protein into host cells. Recently, we have used this assay to show that several candidate DC3000 effectors are translocated into plant cells (Schechter, Roberts, Jamir, Alfano and Collmer, unpublished). This assay will continue to be important in defining new effectors and in the further dissection of the type III secretion signals present in DC3000 effectors.

4.4. N-terminal Characteristics to Identify ORFs that Encode TTSS Substrates

It was clear from Hrp box searches on the DC3000 draft genome^{38, 108} (described above) that to identify TTSS substrates efficiently researchers needed search criteria that would identify ORFs encoding type III secreted proteins, instead of just transcriptional units that were regulated with the TTSS. *Yersinia* researchers made significant progress in identifying shared characteristics in the effectors that are secreted from this animal pathogen^{64, 65}. Two recent papers made significant progress at defining characteristics in *P. syringae* TTSS substrates to allow for their identification. Guttman *et al.*⁴⁵ recognized that TTSS substrates have an exceptionally high serine and a low aspartate content. This allowed for a screen of ORFs downstream of typical Hrp promoters for these characteristics and predicted whether ORFs downstream of Hrp boxes encoded TTSS substrates. This paper identified many candidate DC3000 effectors and two of these, named HopPtoO and HopPtoP, were confirmed to be effectors⁴⁵. In the second paper, Petnicki-Ocwieja *et al.*⁸¹ screened ORFs downstream of typical and moderately atypical Hrp promoters for ORFs that possessed horizontal transfer indicators such as differential G+C content and/or proximity to mobile DNA. Direct testing of 11 of these demonstrated they were TTSS substrates⁸¹. By compiling these TTSS substrates with all of the known *P. syringae* TTSS substrates, the majority of which were Avr proteins, shared biophysical characteristics were identified in their N-termini. These characteristics included the following: An isoleucine, leucine, valine or proline present in position 3 or 4; no aspartate or glutamate within the first 12 amino acids; a high percentage of serine and/or glutamine residues and an overall amphipathic character in the first 50 amino acids. These characteristics were the basis for an algorithm that enabled a bioinformatic search of the DC3000 genome for ORFs that encoded products that shared these characteristics. Importantly, this search was independent of the presence of Hrp promoters and resulted in an enriched list of 32 ORFs that

shared these N-terminal characteristics. Two of these, thus far, were shown to be type III secreted⁸¹.

4.5. Identification of DC3000 Type III Chaperone Genes Based on their Proximity to Effector Genes

Several TTSS effectors from animal pathogens utilize specific chaperones to facilitate their secretion^{35, 80}. These proteins share some characteristics including a predicted α -helical structure in their C-termini, a small size (<20 kDa), an acidic pI (5–6) and the genes that encode them are generally located adjacent to their cognate effector genes. Type III chaperones often prevent aggregation and/or proteolysis of their cognate effectors within the bacterial cell. However, why certain effectors require chaperones while others do not remains unknown. Interestingly, there is mounting evidence that some type III chaperones enable their effectors to better compete for the type III secretion apparatus^{19, 98}. Thus, they may be involved in setting up a hierarchical secretion of effectors from TTSS-containing bacteria. The presence of type III chaperones in bacterial plant pathogens has only recently been discovered^{40, 99}. In *P. syringae* 61, ShcA (named for specific *Hop* chaperone) acts as a type III chaperone and facilitates the secretion of the HopPsyA effector⁹⁹. ShcA and HopPsyA are also present in DC3000, and we have confirmed that these proteins interact with each other as they do in *P. syringae* pv. *syringae* 61 (Guo and Alfano, unpublished). We identified several other putative type III chaperone genes flanking effector genes represented in the nucleotide databases, including several from DC3000^{5, 99}. The availability of the DC3000 genome sequence and the identification of a large number of effector genes allowed us to perform a genome-wide search of the regions flanking effector genes for the presence of ORFs that shared characteristics of type III chaperone genes (Guo, Chancey, Janes and

Table 4. DC3000 type III chaperones and candidate chaperones.

Chaperone or ORF name	Size (kDa)	pI	Effector or ORF name	References
ShcA _{Pto}	14.4	5.3	HopPsyA _{Pto}	[100]
ShcM	18.0	5.3	HopPtoM	[11], [100]
ShcS1	15.6	5.5	HopPtoS1	Alfano, unpublished
ShcV	14.7	6.5	HopPtoV	Alfano, unpublished
ShcF	15.6	6.1	HopPtoF	Tang, unpublished
PSPTO1369	19.9	6.8	HopPtoN	[100]
PSPTO1376	14.6	5.3	AvrE	[100]
PSPTO4589	17.0	5.9	PSPTO4588	Alfano, unpublished
PSPTO4598	17.2	5.3	PSPTO4597	Alfano, unpublished

Alfano, unpublished)²³. We found that at least nine candidate chaperones are present in DC3000 (Table 4). Recently, it was confirmed that ShcM did act as a chaperone for HopPtoM¹¹. In unpublished research from the laboratories of the authors it was also recently shown that ShcS1, ShcV and ShcF act as type III chaperones for the effectors HopPtoS1, HopPtoV and HopPtoF, respectively.

5. PERSPECTIVES ABOUT THE RESEARCH ROAD AHEAD

The completed genome sequence of DC3000 should greatly facilitate progress on understanding bacterial pathogenicity. Moreover, because we also know the genome sequence of *Arabidopsis*, a host of DC3000, the DC3000-*Arabidopsis* pathosystem should continue to be of major importance in dissecting the molecular mechanisms of pathogenesis. Much progress was made in identifying additional effectors of the DC3000 TTSS with the DC3000 draft genome. While little is known about the roles of these effectors in plants, several key papers have been published this year suggesting that at least a subset of them suppress the plant innate immune system (Figure 2). Two papers showed that AvrRpt2, a type III effector from *P. syringae* P4 tomato JL1065, caused the elimination of the *Arabidopsis* RIN4 protein resulting in the suppression of plant defenses^{9, 68}. Evidence supports that AvrRpt2 is an active cysteine protease⁸. Thus, AvrRpt2 joins a growing list of effectors in bacterial plant pathogens that are cysteine proteases^{50, 92} indicating that degradation of proteins involved in signal transduction pathways may be a strategy that plant pathogens employ to circumvent the plant's innate immune system. A particularly well-defined example of this has been recently demonstrated for the *P. syringae* phaseolicola AvrPphB cysteine protease⁹¹. As far as DC3000 effectors, there has been recent progress on three effectors: AvrPtoB was recently shown to suppress programmed cell death in plants and yeast¹; AvrPto was shown to suppress plant cell wall-based plant defenses⁴⁶; and the effector HopPtoD2 was shown to be an active protein tyrosine phosphatase that suppresses plant defenses^{21, 34}, and inhibits a plant MAP kinase pathway that functions in innate immunity³⁴. We still have a long way to go before we have a good understanding of how each of these effectors function to suppress plant defenses and with greater than 30 DC3000 effectors apparently being translocated into plant cells there is still much research to do to better understand their effects on plants.

One thing that is becoming apparent from the recent studies on effectors is that their defense suppression activities appear to be acting on two different types of plant defense responses, the general nonhost defense response and the defense response triggered by Avr proteins translocated into plant cells by bacterial plant pathogens (Figure 2). Plants can recognize conserved molecules

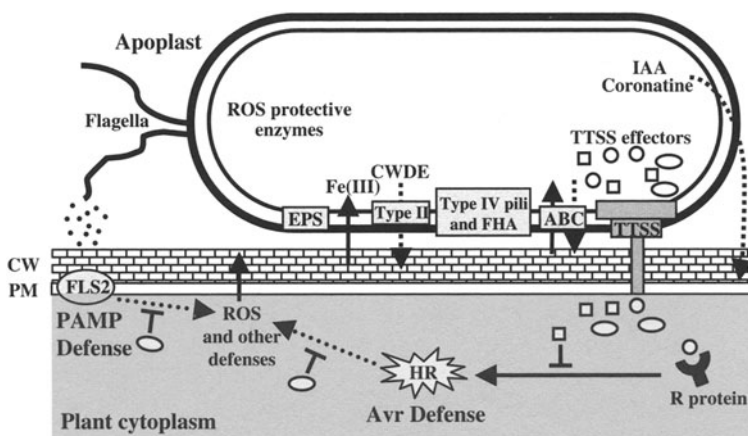


Figure 2. Model of *P. syringae* plant pathogenesis based on the DC3000 genome adapted from Buell *et al.*²³. Central to *P. syringae* pathogenesis is the TTSS and the effectors (depicted by circle, square and oval objects) that it translocates into plant cells. An effector protein (i.e., Avr protein) that is recognized by a resistance protein (R protein) of the plant innate immune system rapidly triggers defense response including the HR and ROS (referred to here as Avr defense). Another part of the plant innate immune system enables the plant to recognize conserved molecular markings on microorganisms referred to as PAMPs (referred to here as PAMP defense). The *Arabidopsis* FLS2 receptor kinase recognizes bacterial flagella and induces the production of ROS and other defenses. There have been several recent reports demonstrating that *P. syringae* effectors are capable of suppressing plant defense responses (see text for examples). Thus, even though *P. syringae* effectors are not well understood, a major role for them appears to be suppression of the plant innate immune system. Other virulence factors likely to contribute to *P. syringae* pathogenesis are as follows: The phytoxin coronatine; the phytohormone IAA; ABC importers and exporters; adhesion mechanisms such as type IV pili and FHA; a type II secretion system, which is likely to secrete cell wall-degrading enzymes (CWDE); siderophores and siderophore receptors for iron acquisition; the exopolysaccharide alginate and levan; and a multitude of enzymes that may protect *P. syringae* from ROS.

produced by microorganisms¹⁸. These signals are analogous to the signals called pathogen-associated molecular patterns (PAMPs) by researchers that study the mammalian innate immune system⁷¹. The other type of plant defense is triggered by molecules (i.e., Avr proteins) produced only by pathogens. Both these responses are perceived by receptor-based recognition systems and induce similar defenses. However, the Avr-induced defense pathway also triggers the hypersensitive cell death, which is not known to be induced during PAMP recognition⁴⁷. The plant defenses that AvrPto suppresses can be induced by nonpathogens^{22, 46} and these are likely triggered from PAMP recognition, and the MAP kinase pathway that HopPtoD2 inhibits is induced by flagellin (i.e., a PAMP) recognition via the FLS2 receptor kinase^{7, 34, 42}. The defense suppression that occurs via AvrRpt2 and AvrPtoB appears to be the Avr-induced

defense type because these suppress the HR induced by specific Avr proteins. These effectors also may suppress PAMP-induced defenses, but this remains to be tested. Perhaps, we should consider these defense systems as part of the same innate immune system that recognizes both conserved molecules (PAMPs) and molecules specific to pathogens (Avr proteins). Future work on the targets of the many effector proteins in DC3000 may provide additional insights about how these proteins disable the plant innate immune system.

The sequencing of the DC3000 genome clearly shows that *P. syringae* pathogenesis is a multifactorial process due to the large number of virulence factors encoded in the genome (Figure 2). We have made progress on some of these and understand some of their effects on plants, while others have only recently been discovered and are not well understood. For example, we do not know if the type II protein secretion system is functional, or what substrate is traveling this secretion pathway. And it is unknown whether any of the ABC translocators contribute to virulence. Clearly, much research needs to be carried out on the newly identified virulence factors present in DC3000. Another lesson from the DC3000 genome is how highly redundant many of the virulence factors appear to be. For example, there are greater than 30 TTSS effectors and many of these appear to be functionally redundant. It is not surprising that most DC3000 effector mutants have weak virulence phenotypes. From the genome sequence, it is also apparent that ROS protective enzymes, siderophores and their receptors, adhesion factors and ABC transporters are redundantly encoded in the DC3000 genome. To decipher each of their contribution to virulence will likely require the construction of DC3000 polymutants. Thus, goals for researchers studying *P. syringae* are quite clear due in large part to the availability of the DC3000 genome sequence. The next few years of research on *P. syringae*–plant interactions promises to reveal much about molecular mechanisms of bacterial plant pathogenesis.

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COMPARATIVE GENOMICS OF FOUR *PSEUDOMONAS* SPECIES

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1. INTRODUCTION

The genus *Pseudomonas* is one of the most diverse bacterial genera, containing over 60 validly described species, isolated from sources ranging from plants to contaminated soils and water to human clinical samples. They are obligate aerobic chemoorganotrophs capable of living on a wide range of aliphatic and aromatic carbon compounds. Not surprisingly, the genus *Pseudomonas* is also phylogenetically rather heterogeneous, containing several subgroups³.

The genome sequence of bacteria from four different *Pseudomonas* species has recently been determined. Each of these species represent a major subcluster of the authentic *Pseudomonas* species. *P. aeruginosa* is a ubiquitous

environmental bacterium that is one of the top three causes of opportunistic human infections, and was the first species sequenced²⁹. *Pseudomonas putida* is a versatile saprophytic organism that has attracted considerable attention due to its potential for biotechnological applications²³. The genomes of *P. aeruginosa* and *P. putida* contain many similar genes, but exhibit different codon usage³⁵. *Pseudomonas syringae* pathovar tomato DC3000 is an organism that is phytopathogenic for tomato and *Arabidopsis thaliana*⁶. Finally, *Pseudomonas fluorescens* is a plant growth-promoting rhizobacterium, and the sequence of *P. fluorescens* SBW25 has been finished and is available from the Sanger Center webpage*, although at the time of writing this genome sequence has not been published.

The goal of this study is to compare some of the properties of the genome of these organisms. First, the genomes will be compared at the DNA sequence level. Then gene conservation will be examined, followed by an analysis of sigma 70 factors. Finally, the proteomes will be compared in terms of differences in protein function, based on a keyword analysis. At all levels examined—that is, the DNA sequence, genes encoded and protein function, *P. syringae* appears to stand out amongst the four *Pseudomonas* species.

2. A GLOBAL VIEW OF THE *PSEUDOMONAS* GENOMES

Pseudomonad genomes typically have sizes between 6 and 7 Mbp (see Table 1); when compared to the more than one hundred sequenced bacterial genomes, only a handful of them are larger than the *Pseudomonas* genomes (e.g., the *Bradyrhizobium japonicum* genome of 9.1 Mbp)[†]. *P. aeruginosa* was the largest bacterial genome at the time when it was sequenced, although now the genome appears closer to the typical size for an environmental bacteria. Among the *Pseudomonas* genomes sequenced to date, the *P. aeruginosa* genome has the lowest AT-content (33%), whilst the other three strains have 38–42% AT (see Table 1). All four of the *Pseudomonas* genomes contain around 5,450 (+/– 100) genes, and between 4 and 7 rRNA operons (see Table 1).

The fraction of repeated sequences, as shown in Figure 5.1A, is a simple yet important measure of a genome's properties. Global direct and global inverted repeats were calculated as described elsewhere¹⁴, and the fraction of the genome with 80% or more identity to sequences elsewhere in the genome

*http://www.sanger.ac.uk/Projects/P_fluorescens/.

†A current listing of sequenced genomes can be found at the GenomeAtlas web resource <http://www.cbs.dtu.dk/services/GenomeAtlas/>.

Table 1. Characteristics of four sequenced *Pseudomonas* genomes. The size of *P. fluorescens* SBW25 genome is an estimate based on the current assembly from the Sanger Center.

Organism	Size (bp)	% AT	Number of genes	Number of rRNA operons
<i>P. aeruginosa</i> PA01	6,264,403	33.4	5,566	4
<i>P. fluorescens</i> SBW25	6,703,654	40.0	5,480	5
<i>P. putida</i> KT2440	6,181,863	38.5	5,350	7
<i>P. syringae</i> pv. tomato DC3000	6,397,126	41.6	5,471	5

is shown in Figure 1A. Global repeats include duplicated regions of the chromosome, such as multiple rRNA clusters; the average level of global repeats in bacterial genomes is around 4%. Of the four genomes, only *P. syringae* differs significantly in global repeats from the average of 150 sequenced genomes (solid black bars in Figure 1). The larger amount of global repeats in *P. syringae* are in part reflective of the larger amount of transposable elements in this genome⁶.

P. aeruginosa has a higher level of local repeats than the other *Pseudomonas* genomes. This is in part due to the lower AT content (33%) than the other genomes. As the base composition deviates more from 50%AT content, the chances of finding local repeats increases. Consistent with this is the relatively low local repeat levels of *P. syringae*, which has the AT content closest to 50% (e.g., 42%AT) of the four species. Local repeats can play a role in DNA mutations, although for GC-rich organisms like *Pseudomonas*, the role of local repeats is less clear. Finally, all the *Pseudomonas* genomes have a higher fraction of direct repeats than inverted repeats (both globally and locally); this is a trend seen in most bacterial genomes^{1, 14}.

Pseudomonas genomes also differ from many other bacterial genomes in that they have a bias toward underrepresentation of purine stretches, whilst on average most bacterial genomes tend toward overrepresentation of purine stretches, as shown in Figure 1B. In contrast, alternating pyrimidine/purine stretches tend to be overrepresented in the *Pseudomonas* genomes. This bias is likely due to enviornmental factors^{33, 35}, and it is interesting to note that many of the bacteria with fewer purine stretches than expected are soil bacteria.[‡]

[‡]Data not shown, but a list of bacterial genomes sorted by this bias can be found on our website <http://www.cbs.dtu.dk/services/GenomeAtlas/database-tables.php>.

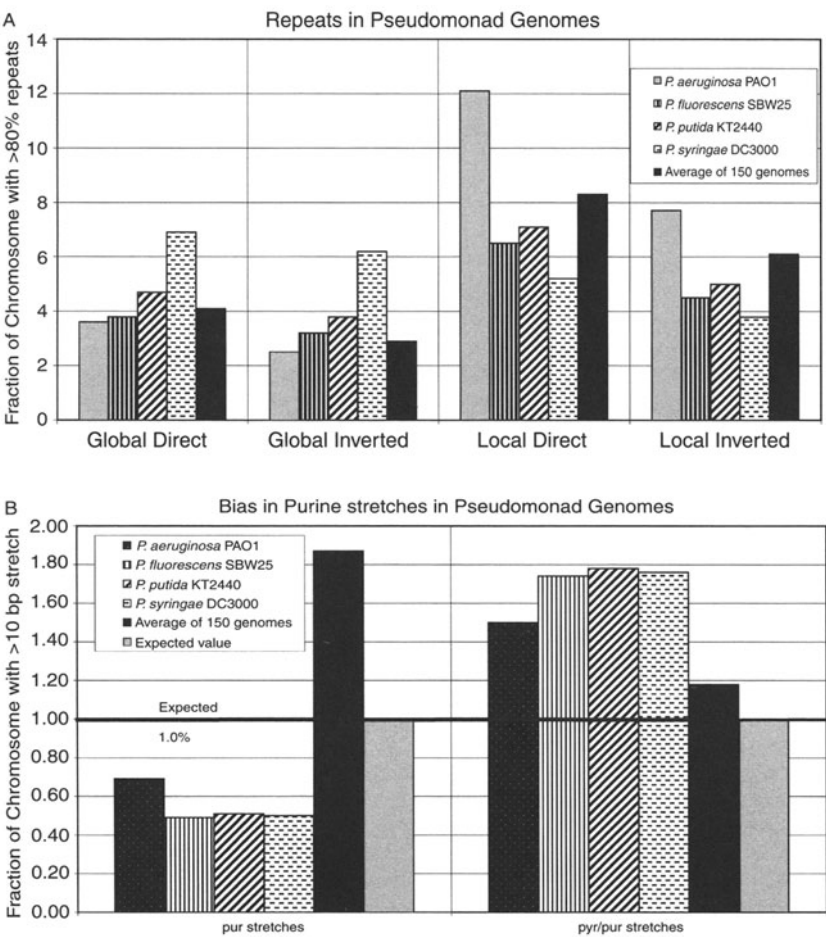


Figure 1. Global DNA properties of *Pseudomonas* genomes. A. Direct and inverted DNA repeats. B. Bias in purine and pyr/pyr stretches.

2.1. Genome Alignment

The complete DNA sequences of three *Pseudomonas* genomes were aligned, using the Artemis Comparison Tool (ACT), downloaded from the Sanger Center website. In Figure 2 the *P. putida* is in the middle, aligned against the *P. aeruginosa* genome (top) and the *P. syringae* genome (bottom). As can be seen from this figure, there are many rearrangements—in particular near

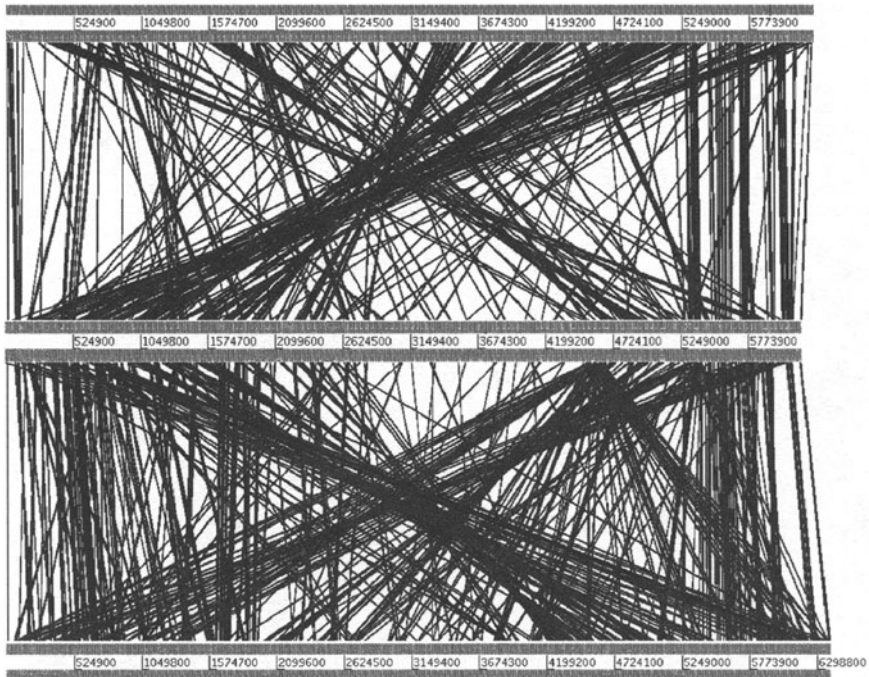


Figure 2. Alignment at the DNA level of the *P. aeruginosa* (top), *P. putida* (middle) and *P. syringae* (bottom) genomes. Blast hits with an S-score of 750 or greater are shown.

the replication origin (e.g., near either edge of the figure), with less sequence conservation near the replication terminus (toward the middle of the figure). Inversions around the origin of replication (often between two rRNA operons) are common in *Pseudomonas* genomes; for example, the *Pseudomonas aeruginosa* PAO1 genome sequenced strain is known to have undergone an inversion around the replication origin, relative to other PAO1 isolates²⁹. Thus, although many of the genes are conserved within the different species, the relative location within the chromosome is likely to be quite different.

2.2. Gene Comparison

In addition to the published genomes available from GenBank, there are also available several *Pseudomonas* genome sequences from various strains that are not in one contiguous piece, but likely contain all the proteins. Using BLAST², an all-against-all comparison of seven publically available *Pseudomonas* genomes has been constructed. For a systematic comparison, potential genes for each genome was determined using EasyGene¹⁵, with an

R-value of 2 as a cutoff. All open reading frames (ORFs) were translated into their amino acid sequence for the BLAST comparison.

A BLAST database was generated for each proteome and all proteomes were aligned against each of the databases. This resulted in a matrix of BLAST reports from which alignments were counted. Only alignments with a minimum of 80% overlapping amino acids and having an expected value (*E*-value) below 1×10^{-5} were included. The parameters were set such that only the best hit per gene was reported.

Based on these results, the table shown in Figure 3 was constructed, which visualizes the homology. Each count of homology was calculated relative to the number of genes in each of the genomes. That is, having 400 BLAST hits from a total of 4,000 proteins gives 10% homology. For homologies *between* genomes, the highest possible number of homolgy (100%) was illustrated with a dark gray color, whilst the white color represents

Comparison of Pseudomonas genomes

ALR=80%, E-value < 1x10-5

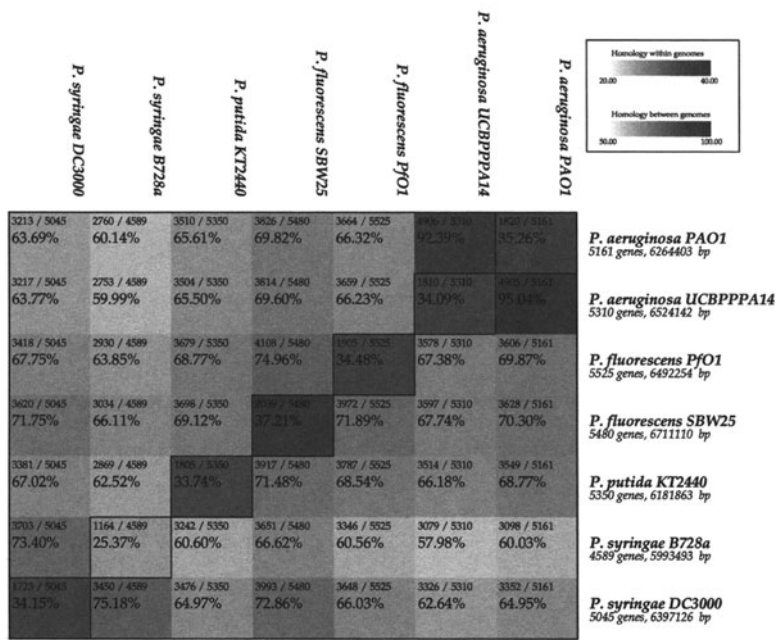


Figure 3. Comparison of genes encoded by seven different *Pseudomonas* genomes. See text for details.

50% homology. For homologies *within* genomes, the scale ranges from 20 to 40%. Within-genome homology is observed down the diagonal of the BLAST table, whilst between-genome homology is observed on each side of the diagonal. Note that the percentage of homology is not identical in A compared with B as when B is compared with A. This is due to the fact that not all genomes have the same number of proteins. That is, on each side of the diagonal, similar hit counts are observed though the percentage will vary slightly due to small differences in gene number.

Inspection of Figure 3 shows that the two *P. aeruginosa* strains are quite similar to each other, both having in common more than 90 % of the proteins, as described using the above criteria. However, the two *P. syringae* strains have only about 70% of the proteins in common, and the two *Pseudomonas fluorescens* strains contain less than 60% of the same proteins. Thus based on this limited set of genomes, it appears that the genomic diversity within the *P. aeruginosa* strains are less than that of the other *Pseudomonas* species. An experimental approach has shown that only about 10% of the genes vary in many *P. aeruginosa* strains. Based on the results described here, this number is likely to be much higher for the other *Pseudomonas* genomes. It is also worth noting that the two *P. syringae* strains seem to differ the most from the other genomes, with an average of around 45% homologs; this can be visualized by the lighter color in the bottom two rows in Figure 3. Thus as with the repeats, the *P. aeruginosa* and *P. syringae* genomes stand out as distinct from the other three, under different circumstances.

2.3. Sequence Profile Search for ECF-Type Sigma Factors

There is a great difference in the ability of various microorganisms to adapt to changes in their environment. At one end of the scale we find parasites and obligate symbiotic lifeforms, which have evolved to live in one very constant environment—for example, inside another cell. *Pseudomonas* is at the other end of the spectrum, consisting of freeliving species with the ability to adapt to a large number of very different environments. This adaptation to different environments is to a large extent attained through the expression of different sets of genes under different circumstances. In bacteria part of this regulation is often controlled by different σ -factors, which can initiate transcription through the recognition of different upstream elements. The number of σ -factors in an organism can be considered as one measure of its adaptability. This number varies from 1 for pathogens with small genomes, such as *Mycoplasma genitalium*¹², to 65 for *Streptomyces coelicolor*⁴. In the *P. aeruginosa* and *P. syringae* genomes there are 24 σ -factors. A detailed comparison of σ -factors in these two genomes found 13 extracytoplasmic (ECF) σ -factors with homology to *E. coli* FecI, which is involved in iron acquisition¹⁸.

The σ^{70} related σ -factors are both the most abundant and the most diverse family. The evolutionary unrelated σ^{54} -factors are usually present in a single copy per genome, and have not been included in this analysis, although they are discussed in another chapter in this book, dedicated to sigma factors. The σ^{70} family encompasses the primary and the ECF σ -factors. Little is known about most of the ECF σ -factors, except that many of them are cotranscribed with their negative regulators, known as anti- σ -factors. These are often transmembrane proteins serving and thus thought to act as sensors of the extracellular environment²⁰.

Starting with a single ECF σ -factor sequence (SigY from *Bacillus subtilis*), PSI-BLAST² was used for identifying an initial sequence set of putative ECF σ -factors by searching against a database of all ORFs longer than 300 bp from 100 bacterial genomes. A better quality multiple alignment of these sequences was constructed using ClustalW³² and a profile HMM was constructed using hmmbuild from the HMMER package⁹. The resulting HMM was used for searching the database again using hmmsearch to identify a more accurate ECF set. This procedure, starting from the ClustalW alignment, was iterated until convergence was achieved.

Using this method, we identified σ^{70} related σ -factors in the genomes of seven *Pseudomonas* species in Figure 3. The simplest possible analysis one can perform is to simply count in each genome the number of genes encoding σ^{70} /ECF transcription factors, and the results are shown in Figure 4.

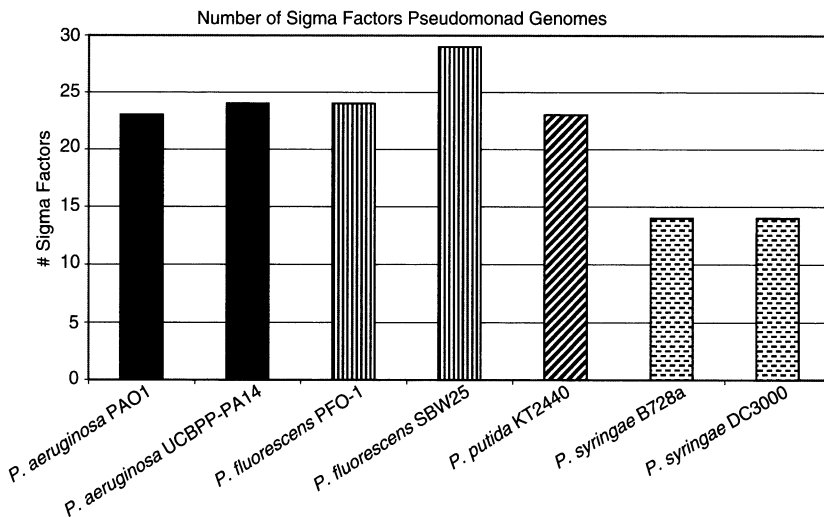


Figure 4. Comparison of genes encoded by seven different *Pseudomonas* genomes. See text for details.

Although for genomes from the first three of the *Pseudomonas* species shown we find at least 23 σ^{70} related σ -factors, for both *P. syringae* genomes, only 13 σ -factors are found. This much lower number of σ -factors in *P. syringae* compared to the other *Pseudomonas* is very surprising as the loss of even a single σ -factor should result in a number of genes no longer being available to the cell as they cannot be transcribed. Thus, once again, *P. syringae* stands out as unique amongst the four species compared.

2.4. Phylogenetic Analysis of ECF σ -factors

To analyze which specific groups of σ -factors have been lost in *P. syringae*, we made a tree of all *Pseudomonas* σ^{70} -factors. Using the self-consistent profile HMM constructed above, hmalign was used to align all 83 σ^{70} /ECF sequences from the four *Pseudomonas* species to the model. From the resulting alignment, the most conserved columns were extracted, namely those corresponding to match states in the profile HMM. From this core alignment, pairwise evolutionary distances were estimated using the protdist program from the PHYLIP package¹⁰. Finally, an evolutionary tree was constructed using the UPGMA algorithm (Figure 5). Where infinite evolutionary distances were reported by the protdist program, a distance of 99 (a very large distance) was used.

All but one of the σ -factors missing in *P. syringae* are of the ECF type; only a small number of these have previously been associated with pathogens and symbionts²¹. This fits very well with *P. syringae* being a plant pathogen, but may at first seem to contradict the high number of ECF σ -factors found in the pathogenic *P. aeruginosa*. However, *P. aeruginosa* is only an opportunistic pathogen and lives freely in soil too.

The loss of FliA, one of the four major members of the σ^{70} family, is more surprising. FliA regulates the expression of the flagellar as well as chemotaxis genes. Since *P. syringae* are known to have flagella²⁶, they might now be under the control of another σ -factor or other regulatory protein. The loss of a σ -factor can either lead to complementation from other systems or loss of function of the genes regulated by the σ -factor¹⁷. While such complementation must have taken place for the flagella, it is likely that some chemotaxis genes have simply been lost. Similarly, the loss of 10 ECF factors is likely to result in loss of function and possibly pseudogenes in the *P. syringae* genome.

The other three major σ -factors (RpoD, RpoH and RpoS) are present in all four *Pseudomonas* species, and form very clear clusters. In all three clusters *P. syringae*, *P. fluorescens* and *P. putida* form a clade with *P. aeruginosa* separating from them earlier in evolution. This is consistent with the 16S rRNA phylogeny. Phylogenies derived from these three σ -factors, however, disagree on the evolutionary relationship between *P. syringae*, *P. fluorescens* and *P. putida*.



Figure 5. Phylogenetic tree of 83 *Pseudomonas* σ^{70} -factors, from the genomes in Table 1. Single-letter abbreviations are used for each genome: A = *P. aeruginosa*, F = *P. fluorescens*, S = *P. syringae*, and P = *P. putida*.

3. SYSTEMATIC FUNCTIONAL COMPARISON OF COMPLETE GENOMES

Since it is generally not known which sets of genes in a genome are under the regulation of which σ -factors, it is hard to put the above results in a functional context. We approach this problem through a systematic search for differences in the functional content of the genomes.

To accomplish this we extracted all ORFs longer than 300 bp from each of the four genomes and translated them to their corresponding amino acid sequences. From these, BLAST databases were constructed, and all pairwise comparisons were performed using gapped BLASTP with low complexity filter enabled². Each of these sets was also searched against the SWISS-PROT database⁵. The SWISS-PROT database contains proteins that have been experimentally verified and have a known function. Hence, a good BLAST hit to SWISS-PROT means the chances for knowing the true function of the protein are much greater, since a similar protein has already been characterized in the literature.

Based on these similarity searches, all *Pseudomonas* protein sequences with significant matches (E -value better than 10^{-6}) to SWISS-PROT were identified. For each of these sequences functional information in the form of SWISS-PROT keywords was transferred from the best match. As only sequences with significant matches to SWISS-PROT are used in the subsequent analysis, the large number of random ORFs do not present a problem.

Subsequently, the protein sequences unique to each of the four *Pseudomonas* (but possibly present in other organisms) were identified. An E -value of 10^{-9} was used as cutoff for the BLAST matches for identification of homologs in both the other *Pseudomonas* and in SWISS-PROT. Similarly, the proteins encoded by each genome having homologous genes in all of the three other species were identified, as were the proteins showing homology to each combination of two species.

For each such set of protein sequences, a systematic search for keywords overrepresented, compared to the reference genome, was performed using hypergeometric statistics. The probability of observing a given keyword exactly x times in a sample is

$$P(x) = \frac{\binom{m}{x} \binom{N-m}{n-x}}{\binom{N}{n}},$$

where n is the size of the sample, N is the size of the pool (i.e., the total number of sequences with significant matches to SWISS-PROT), and m is the

number of sequences labeled with the keyword. Using the *R* statistics package, we have calculated the cumulative probability that each keyword would, at random, occur as many or more times than the actual observed number, that is, the level of significance that a given keyword is overrepresented in a particular partition.

The significance of each keyword also varies depending on which genome is chosen as reference. While this is not a problem when studying proteins present in only one of the *Pseudomonas*, it presents a problem when studying proteins present in several of the genomes.

3.1. Biological Interpretation of the Keywords

Table 2 lists the significantly overrepresented keywords for each of the four genomes from Table 1. Interestingly, *P. syringae* is functionally most dissimilar to the other *Pseudomonas* species. This agrees well with the much lower number of σ -factors present in its genome, and the fewer genes in common with other *Pseudomonas* genomes in Figure 3.

The three most overrepresented keywords in *P. syringae* are all related to transposons: “DNA recombination,” “Transposition,” and “Transposable element.” Indeed, inspection of the *P. syringae* genome reveals the presence of 13 different families of transposons. The keyword “Plasmid,” which may appear to be non-intuitive for chromosomal genes, could indicate that a large number of the genes only found in *P. syringae* may have been incorporated from plasmids.

The final keyword significantly overrepresented among *P. syringae*-specific genes, “Tellurite resistance,” makes biological sense too, as tellurium resistance has indeed been reported for *P. syringae*⁸. The toxicity of tellurite is largely due to it being oxidative. The resistance mechanism involves reduction of the oxidative tellurite to a less harmful compound and subsequent extrusion³¹.

In *P. aeruginosa*, the keyword “Virulence” and “Plasmid” are found to be overrepresented among genes only found in this genome. The presence of virulence-related proteins should not come as a surprise, as *P. aeruginosa* is known to be an opportunistic pathogen. As was briefly mentioned for *P. syringae*, the presence of proteins typically found on plasmids is also not surprising. In fact, plasmid transfer may very well be the mechanism by which *P. aeruginosa* obtained its virulence genes as such genes are often found on plasmids.

Only one SWISS-PROT keyword, “Methyltransferase,” displayed significant overrepresentation in the set of proteins only found in *P. putida*. This keyword is closely related to one found to be overrepresented among proteins shared by *P. putida* and *P. aeruginosa*—namely “Chemotaxis.” The chemotaxis

Table 2. Significantly overrepresented SWISS-PROT keywords associated with differences among the *Pseudomonas* genomes.

SWISS-PROT keyword	Significance
<i>P. aeruginosa</i>	
Virulence	3.8×10^{-4}
Plasmid	8.5×10^{-4}
<i>P. putida</i>	
Methyltransferase	7.4×10^{-5}
<i>P. syringae</i>	
DNA recombination	$<10^{-9}$
Transposition	$<10^{-9}$
Transposable element	$<10^{-9}$
Plasmid	4.4×10^{-4}
Tellurium resistance	4.6×10^{-4}
<i>P. syringae</i> and <i>P. putida</i>	
Plasmid	$<10^{-9}$
Transposable element	$<10^{-9}$
Hypothetical protein	$<10^{-9}$
Intron homing	$<10^{-9}$
RNA-directed DNA polymerase	$<10^{-9}$
Endonuclease	9.6×10^{-6}
Multifunctional enzyme	3.9×10^{-5}
Nuclease	4.8×10^{-5}
<i>P. syringae</i> and <i>P. fluorescens</i>	
Hypothetical protein	5.5×10^{-4}
<i>P. aeruginosa</i> and <i>P. putida</i>	
Outer membrane	2.1×10^{-4}
Chemotaxis	5.4×10^{-4}
Fimbria	8.4×10^{-4}
All but <i>P. syringae</i>	
Electron transport	3.8×10^{-5}
Arabinose catabolism	1.5×10^{-4}
Arsenical resistance	4.6×10^{-4}
Flavoprotein	7.0×10^{-4}
All but <i>P. aeruginosa</i>	
Serine protease	5.1×10^{-5}
Toxin	1.8×10^{-4}
All but <i>P. fluorescens</i>	
DNA-binding	5.6×10^{-4}
Present in all	
Ligase	1.8×10^{-8}
Protein biosynthesis	5.5×10^{-7}
DNA-binding	2.9×10^{-6}
Transcription regulation	4.0×10^{-6}
Aminoacyl-tRNA synthetase	1.2×10^{-5}
Tricarboxylic acid cycle	1.5×10^{-5}
Ribosomal protein	5.1×10^{-5}

Table 2. *Continued*

SWISS-PROT keyword	Significance
Helicase	1.0×10^{-4}
Lyase	1.6×10^{-4}
Zinc-finger	1.7×10^{-4}
Metal-binding	3.1×10^{-4}
Rotamase	3.4×10^{-4}
Sigma factor	4.0×10^{-4}
ATP-binding	8.1×10^{-4}

mechanism allows bacteria to move in an advantageous way by measuring the gradients of repellants or attractants. At the molecular level chemotaxis involves a number of sensory proteins, methyl accepting chemotaxis proteins (MCP), that measure the concentrations of attractants and repellants over time. MCP bind attractants or repellants, either directly or indirectly, through interactions with periplasmic binding proteins. MCP can be methylated and demethylated by methyltransferases, to alter their activity.

A large number of keywords in Table 2 are associated with proteins common to all four *Pseudomonas*. The vast majority of these would also be expected to be conserved to other species as they stem from essential processes like transcription, translation, and central pathways of the metabolism (e.g., “Tricarboxylic acid cycle”). “Ligase” being the most significant of the keywords can be explained by the involvement of ligases in transcription as well as translation.

The keyword “sigma factor” is one of the more interesting. According to this test the σ -factors are very well conserved in the *Pseudomonas*. Despite *P. syringae* lacking many of the σ -factors found in other *Pseudomonas*, σ -factors are still overrepresented among the proteins conserved in all four *Pseudomonas*.

Among the proteins unique to/absent from each of the four *Pseudomonas*, it is clear that *P. syringae* has both the most significant and largest number of significantly overrepresented keywords. The functionality encoded by the *P. syringae* genome differs from the other *Pseudomonas*—a finding consistent with the study of σ -factors possibly indicating loss of function in the *P. syringae* genome, as well as the BLAST results in Figure 3.

4. GENOME COMPARISON ATLASES

Knowing that functional differences exist among the *Pseudomonas*, it is interesting to examine if these functions are localized within particular regions

unique to the strain. Regions present in one or more of the genomes can be detected in a genome comparison atlas.

4.1. Atlas Visualization

Methods such as “atlas visualization” have previously proven very useful for gaining an overview of local variations in many different genomic features^{14, 25, 28}. We have previously used Genome Atlases to compare the *P. aeruginosa* and *P. syringae* genomes³⁶. Atlases will be used here for comparing genomic features of the four *Pseudomonas* genomes in Table 1, as well as for correlating their differences to DNA properties and the functional characterizations obtained from the keyword analysis.

The matches between the three published *Pseudomonas* genomes were found by aligning all annotated protein coding genes against each other; all ORFs longer than 300 bp were translated and used for *P. fluorescens*. The BLAST matches are visualized as the outer three circles in Figures 6–7 representing each of the organisms against which BLAST searches were performed and a fourth circle representing matches against the SWISS-PROT database⁵. For every protein the negative logarithm of the *E*-value of the most significant match was calculated (imposing a maximum score of 15 for highly significant matches). These values were mapped to the chromosomal location of the corresponding genes. The color scale was reversed so that only lack of hits appear in the circle. This allows us to detect clusters of proteins that are found in only a subset of the analyzed organisms. On each atlas we also show the localization of the most significant keyword (or set of keywords) for the specie in question. These keywords were inferred from BLAST matches to SWISS-PROT and regions were color coded according to their density of genes having the keyword(s).

In addition to these sequences of similarity-based circles, a circle showing the local AT-content is included since it contains local contextual DNA information along the genome. It is visualized using a double-sided color scheme where regions with unusually high or low AT-content are highlighted in red and cyan, respectively. The AT-content is closely correlated to many DNA structural properties²⁵, but is also of interest because foreign DNA acquired through lateral gene transfer often has a different AT-content¹³. A second circle shows the *position preference* (one of the structural parameters least correlated to AT-content), which is a measure of anisotropic flexibility and regions of low position preference are generally correlated with more highly expressed genes^{24, 27}.

The AT-content only contains part of the information that can be encoded by the local base composition. The rest can be represented by a class of parameters called *skews*, which have proven very useful for locating the origin and terminus of replication in bacteria¹⁶.

The skews are independent of the AT-content and instead represent preferences for having G's over C's (the GC-skew) or A's over T's (the AT-skew) on one of the two DNA strands. Both the absolute and relative strength of these skews vary greatly between organisms, as does the orientation of the AT-skew compared to the GC-skew. In the case of *Pseudomonas*, the skews are strong and anti-correlated; they can therefore favorably be combined into a single skew, the *keto skew*, defined as $((G + T) - (A - C))/N^{14, 19}$.

4.2. *P. aeruginosa*

Figure 6 shows a genome comparison atlas for *P. aeruginosa*. The outermost four circles map BLAST hits (or actually the absence thereof) to genes encoded by the *P. aeruginosa* genome. Since so many of the genes are in common for the different genomes, the places where genes are NOT conserved are shaded dark. For example, there are two prophages present in *P. aeruginosa* (between 0.5 and 1.0 Mbp, labeled in Figure 6), which are not found in the other genomes (dark bands in the four outermost circles—note that the first prophage does have a match (hence no dark band) in the *P. syringae* genome). The inner three circles contain DNA structural properties, as described above.

P. aeruginosa separated from the other *Pseudomonas* species earlier in the course of evolution and has since then acquired functions that allow it to be an opportunistic pathogen. In spite of this, the majority of the genes in *P. aeruginosa* have homologs in the other *Pseudomonas* genomes, as can be seen from the relatively few dark lines (representing poor or no matches) in the outer circles. For genes in *P. aeruginosa* but not in the other *Pseudomonas* genomes, two SWISS-PROT keywords were found to be significantly over-represented; “plasmid” and “virulence.” Matches to SWISS-PROT and the other *Pseudomonas*, as well as the position of genes related to the keyword “virulence” can be seen in Figure 6. The keyword “plasmid” is not plotted as it is associated with a large number of different functionalities and is thus found throughout the genome.

Unlike the other *Pseudomonas* species in this study, *P. aeruginosa* is known to be a pathogen infecting humans; it should therefore be possible to see regions of genes that are unique to *P. aeruginosa* and have the keyword “virulence.” The area marked *Type III secretion* is a good example of that. The type III secretion system is known to be a determinant of the virulence of *P. aeruginosa*^{11, 36}. The Hrp type III secretion system that is found in *P. syringae* and *P. fluorescens* is capable of eliciting a hypersensitive response in plant cells⁷. The similarity between the Hrp secretion system and the type III secretion system seen in *P. aeruginosa* is, however, only weak as they serve different functions.

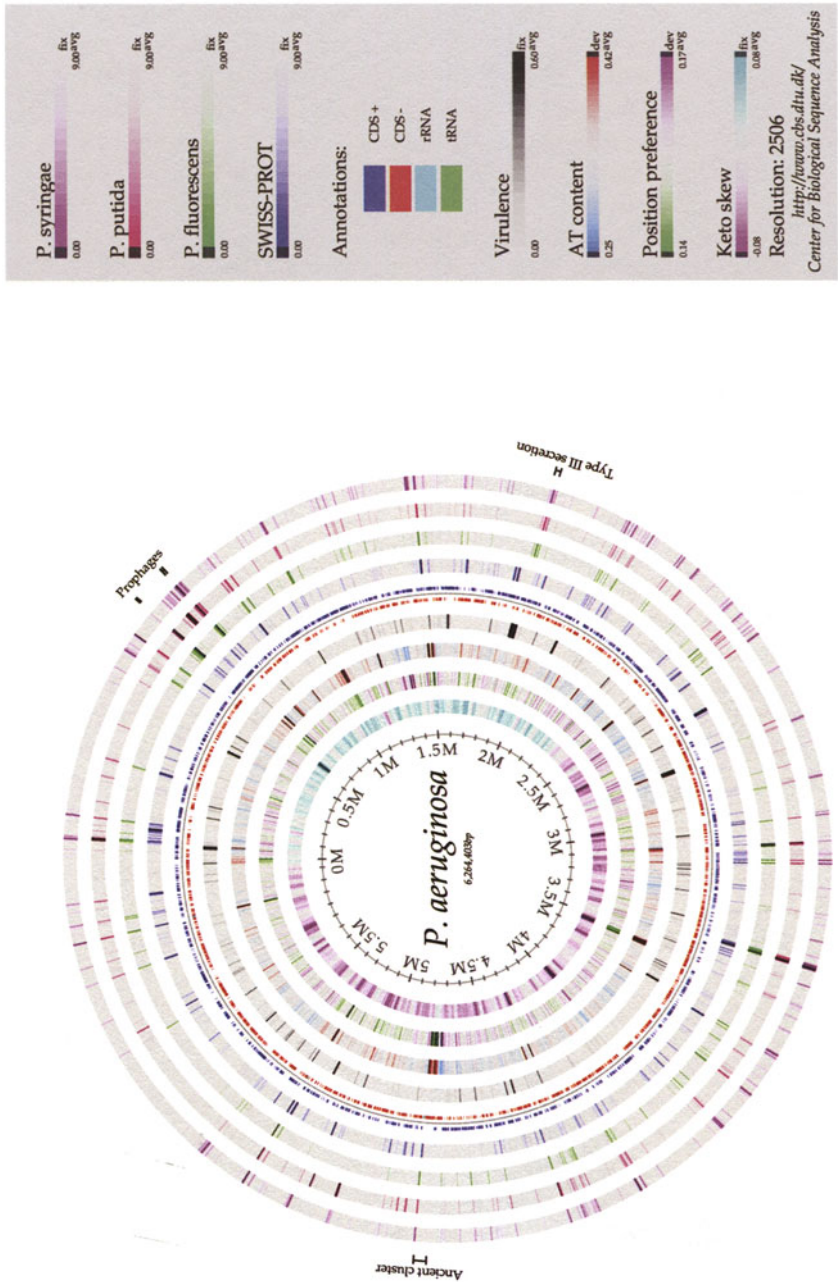


Figure 6. Genome comparison atlas of *P. aeruginosa* PA01. Each of the concentric circles represent genomic features as described in the figure legend on the right, with the outermost circle corresponding to the top-most feature in the legend, and the innermost circle corresponding to the bottom-most feature in the legend. Dark bands in the outer four circles represent the location of genes, which are not conserved in other *Pseudomonas* genomes or in SWISS-PROT (blue circle, fourth lane in the atlas). The inner three circles contain DNA structural properties, as described in the "Atlas Visualization" section of the text.

Two prophages are also labeled on the atlas. One, prophage Pfl, is only found in *P. aeruginosa*, and a putative bacteriophage marked 100 kb upstream appears to have a homolog in *P. syringae* but not in the two other *Pseudomonas*. The two labeled prophage regions have very high AT-content compared to the rest of the genome as is the case for a region at 4.7 Mb. This region was—because of the difference in AT-content—suggested to be a candidate of horizontal gene transfer²⁹. The very low value of position preference also seen in this region indicates that the region is likely to contain highly expressed genes. The corresponding proteins match sequences in SWISS-PROT and the region as such is conserved among *Pseudomonas*. The region is an ancient cluster of genes encoding several ribosomal proteins, subunits of the RNA polymerase, and SecY³⁰. In *P. syringae* and *P. putida* the cluster is located at approximately 0.5 Mb, indicating that the cluster was relocated as part of the proposed chromosomal inversion around the origin²⁹.

4.3. *P. putida*

In *P. putida* one keyword was found to be significantly overrepresented: “Methyltransferase.” Unlike the virulence genes in *P. aeruginosa*, these genes are not found to form clusters within the genome of *P. putida*. A number of other regions found only in *P. putida* are seen in Figure 7. However, as most of these acquired regions contain genes that are not found in SWISS-PROT either, one can only speculate what their functions are. In a few cases where the functionality of some of the genes is known, they have been found to increase the metabolic proficiency of *P. putida*^{23, 34}.

Some of the regions that have matches to SWISS-PROT correspond to *prophages*; these were also found to be connected to viral proteins by the phylome analysis. The *T7 prophage* stands out in particular by also having low position preference and inverted keto skew compared to its surroundings. Incidentally, the only other region with strong skew inversion present in the *P. putida* genome also has very low position preference. It corresponds to a gene encoding a putative 8,682 aa surface adhesion protein, making it the largest of the annotated protein coding genes in *P. putida*.

Many of the inserted regions in *P. putida* (but not the prophages) have a very high AT-content compared to the genome average. This, together with the number of insertions, indicates that *P. putida* is highly susceptible to gene transfer³⁴. The region labeled *Thymidylate insertion* is an example of one such region; it is a 60 kb element inserted in the thymidylate kinase gene. While most of the genes in this region are of unknown function, four adjacent genes have by homology been associated with arsenic/arsenite resistance. In contrast to *P. aeruginosa*, *P. putida* is known to be resistant to both arsenite and arsenate²².

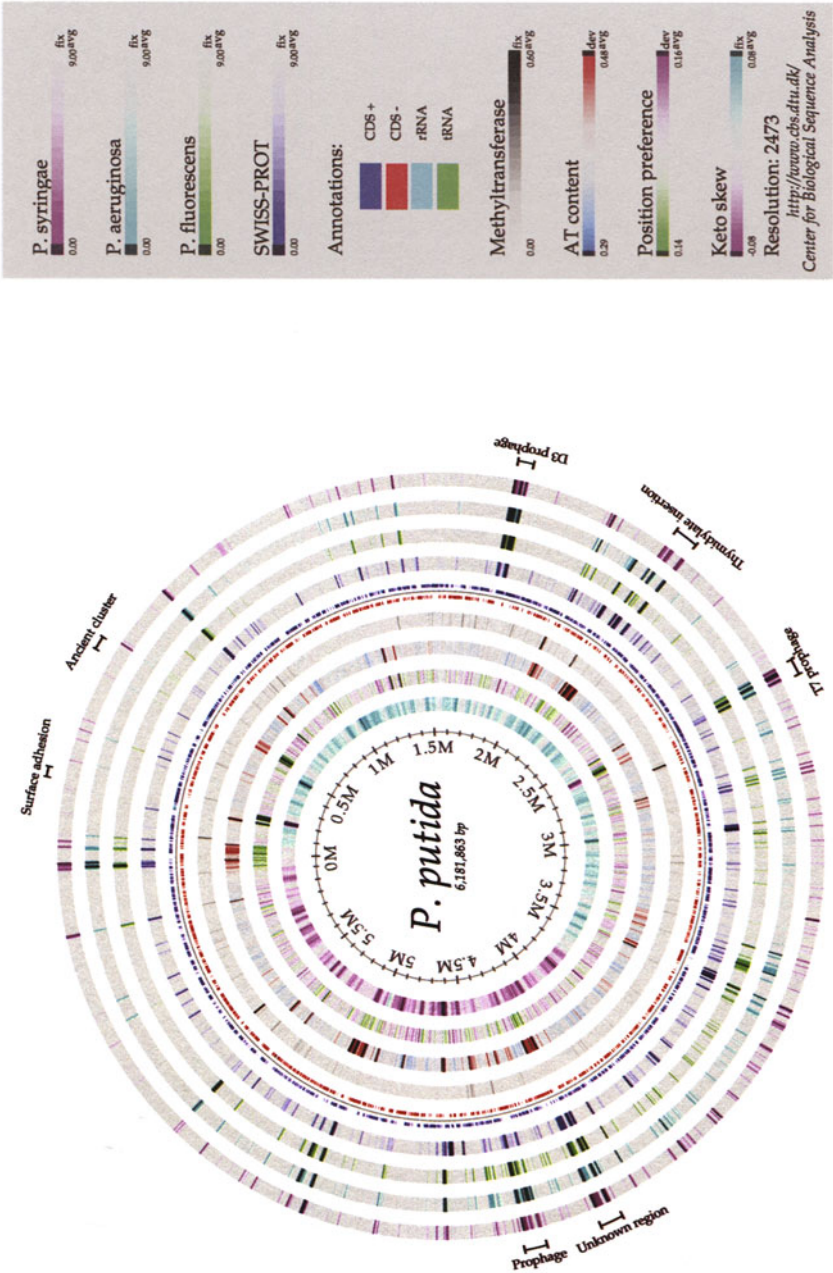


Figure 7. Genome comparison atlas *P. putida* KT2440. The circles are as described in Figure 6.

4.4. *P. syringae*

While *P. putida* is known to be resistant to arsenic containing compounds, the keyword “tellurium resistance” was found to be overrepresented among *P. syringae* specific genes. The genes with this keyword are localized as one gene cluster, which is labeled in Figure 8.

From the large number of significantly overrepresented keywords found for *P. syringae*, it is clear that it is very different from the other *Pseudomonas*. The most significant keywords were “DNA recombination,” “transposable element” and “transposition,” which are closely related and thus shown together as one circle (black circle in Figure 8).

In addition to the genes corresponding to these keywords, a number of genes exist that are found neither in other *Pseudomonas* genomes nor in SWISS-PROT. Similar to what was observed in *P. putida*, these genes are clustered within the genome, although the number of clusters is larger in *P. syringae*. Once again, one can only speculate about the function of these genes.

Two such unknown regions of approximately 100 kb each are found near the *origin* and *terminus* of replication. This pattern is neither seen in *P. putida* nor in *P. aeruginosa*. The positioning of unknown genes near the origin is surprising as this region often contains highly expressed household genes. It is thus intriguing that the region has low position preference (indicating the possibility of highly expressed genes), although it should be noted that this is the case for many other inserted regions as well.

Next to the terminus is located a cluster of genes that are present in *P. putida* too but not in the two other *Pseudomonas* genomes. A number of genes in this region further have similarity to SWISS-PROT sequences from the nitrogen fixating soil bacterium *Rhizobium* *sp.* strain NGR234. However, the function of these is not known, explaining why this group of genes was not discovered in the keyword analysis. Given that *Rhizobium* lives in symbiosis with plants while *P. syringae* is a plant pathogen, we suggest that this region is a likely candidate of lateral gene transfer. This is supported by the high AT-content of the region compared to the genome average as well as by the region being flanked by transposons

Although transposable elements are dispersed throughout the genome, they appear to be especially frequent within the otherwise uncharacterized regions. This is in particular true for the region shown in Figure 9 (this region is labeled *Zoom* in Figure 8). From the zoom it is obvious that the subregions of unknown genes are flanked by genes associated with transposition. The region not only contain genes of unknown function—genes involved in the synthesis of the polyketide coronafacic acid are also located in the region. Furthermore phylome analysis suggests a possible source for these genes as

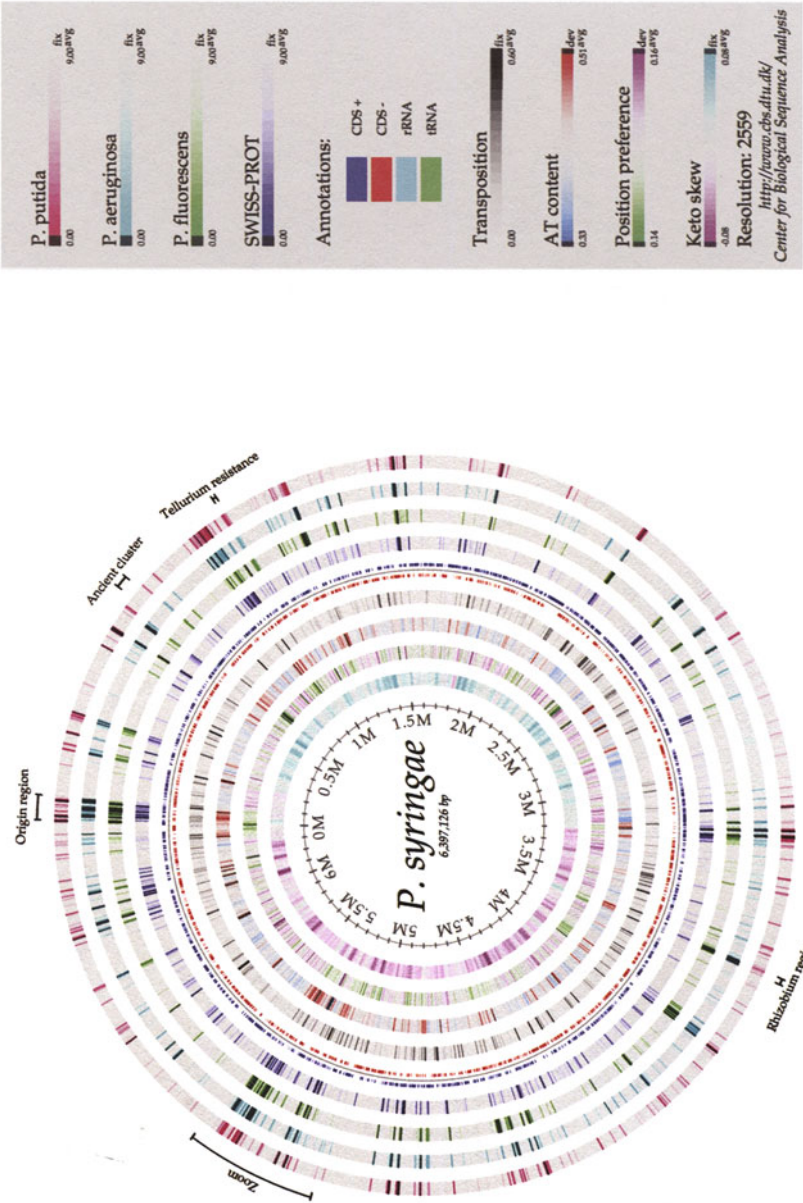
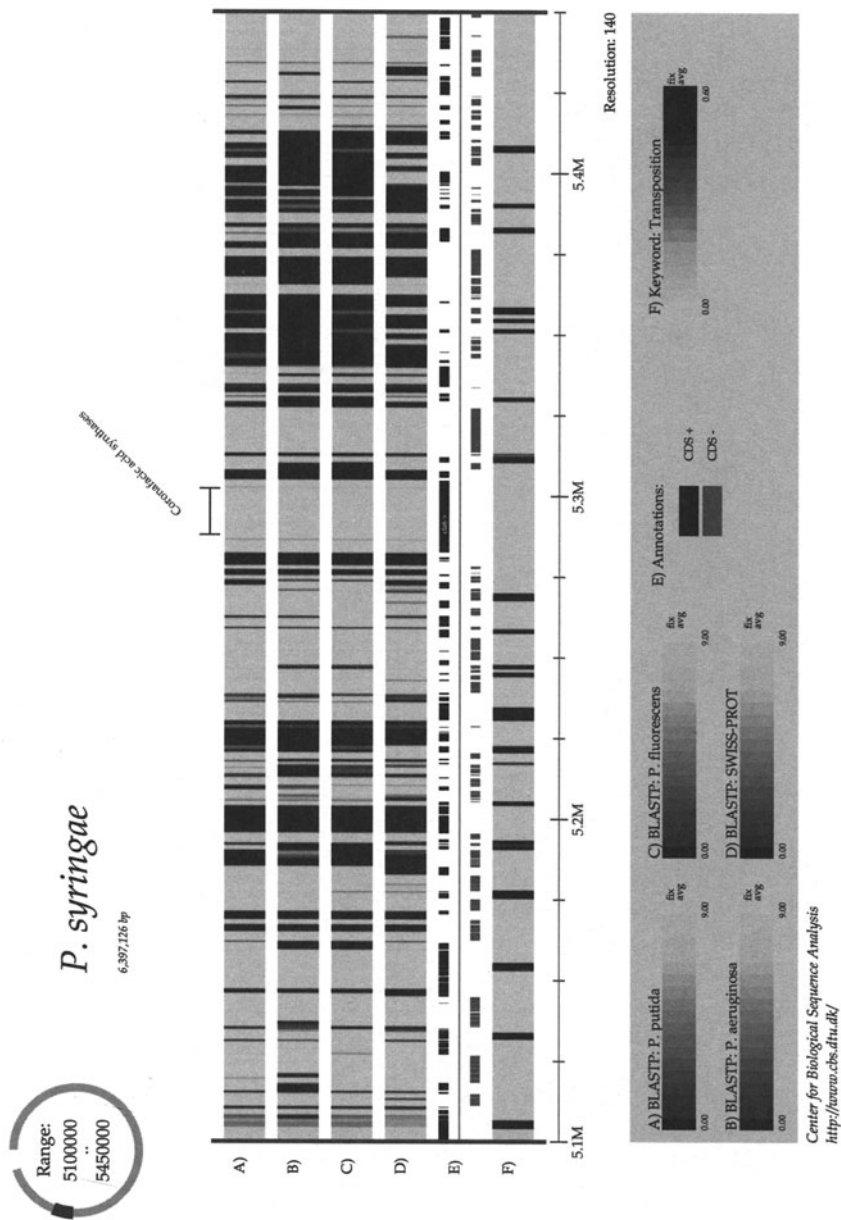


Figure 8. Genome comparison atlas of *P. syringae* pv. tomato DC3000.



they are most closely related to actinobacterial genes. Another observation made from phylome analysis was the relatively high number of *P. syringae* proteins predicted to be of eukaryotic origin, which is possibly due to lateral gene transfer from the host organism.

5. CONCLUSIONS

We have compared the genomes of four different *Pseudomonas* species, using several different approaches, and find that, depending on the features examined, in some ways *P. aeruginosa* stands out as unique amongst the four, but in many other ways, in particular in genome variation, *P. syringae* is significantly different from the other three. It is the fundamental idea of comparative genomics to use the entire genomes as the basis for comparison—however suprisingly few methods have been developed for actually doing it. We present a new approach: a systematic statistical search for differences between the gene functions present in a set of genomes. This allows us to, without prior knowledge, obtain hints about biological properties unique to each organism.

When it comes to gaining an overview of complete genomes, it is difficult to overestimate the importance of good visualization techniques. They enable scientists to discover relations in the data that would otherwise go unnoticed. In the present chapter we have used such a method, the atlas visualization, to both compare the four *Pseudomonas* genomes to each other and relate their differences to DNA structural properties as well as gene functions.

Despite strong phylogenetic support for *P. aeruginosa* being evolutionary separated from the other three *Pseudomonas*, it is clearly *P. syringae* that stands out when one performs the comparison at the genomic level. This suggests that the genome of *P. syringae* must have undergone large changes within a relatively short timespan as part of adapting to a symbiotic/parasitic lifestyle. Many such changes in the *P. syringae* genome are revealed by our analysis, include the loss of many genes and uptake of others, which is likely to be mediated by the large number of transposons dispersed throughout its genome. The same methods, when applied to the *P. aeruginosa* genome, highlighted a number of features related to its pathogenicity, in particular the type III secretory system.

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GENOMICS OF CATABOLIC PLASMIDS

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1. INTRODUCTION

Catabolic plasmids were discovered in the early 1970s by Chakrabarty, working in the laboratory of Gunsalus. These first plasmids were named SAL, OCT and CAM and encoded the catabolism of salicylate¹⁴, octane and other short chain linear alkanes¹⁵, and camphor⁷³ respectively and were all found in strains of *Pseudomonas*. Shortly after, a naphthalene catabolic plasmid NAH was reported by Dunn from the same laboratory²² and then the TOL plasmid, now known as pWW0, was independently discovered and reported by Nakazawa and Yokota⁵⁶, Williams and Murray⁹⁷, and Wong and Dunn¹⁰¹, both also in *Pseudomonas* host strains. Given the remarkable taxonomic schisms that have more recently taken place within the genus of *Pseudomonas* since the advent of 16S rRNA gene sequences, all the host strains for these primary catabolic plasmids have remained solidly within the now much more narrowly defined genus of *Pseudomonas*. But however much the initial breakthrough owes to *Pseudomonas*, it is now clear three decades later that catabolic plasmids are comprehensively distributed across a very broad range of saprophytic soil bacteria.

The substrates whose catabolism is determined by the vast majority of these plasmids are usually what might be considered to be within the more

'exotic' range of substrates used by bacteria, involving hydrocarbons, particularly aromatics, and many man-made chemicals. These are often referred to in the literature as 'xenobiotics', although Wackett and Hershberger⁹³ have recently raised reasonable objections to the use of this term based on our still limited knowledge of the diversity of chemicals produced naturally (i.e., biologically) to which bacteria have had sufficient exposure over a considerable period of time in which to evolve catabolic pathways. For some of these plasmid-encoded growth substrates that have been more extensively studied, such as naphthalene and toluene and the xylenes, the term xenobiotic is clearly a misnomer since they are components of crude oil, itself of biological origin, and natural leakages have allowed continuous bacterial-substrate exposure. Other substrates, for example, many chloro- and nitro-substituted molecules, are more certainly the direct result of the chemical industry and although there might be natural products with some chemical resemblance to them, the compounds themselves have almost certainly been available as potential bacterial substrates for only a few decades. What does seem remarkable is that plasmid carriage of the genes for some of these more 'exotic' substrates including toluene and the xylenes^{47, 78, 101} and naphthalene^{7, 11, 22} seems to be the norm rather than the exception at least within *Pseudomonas* populations. This raises some interesting questions as to why such catabolic genes, in the same manner as antibiotic resistance, should be preferentially found on plasmids rather than stably maintained within the chromosome.

During the early years of catabolic plasmid research, the burden of proof for the existence of a plasmid within a strain relied upon genetic evidence either by the conjugal transfer of the catabolic phenotype between genetically marked, but usually taxonomically close, strains or by the irreversible loss of the phenotype from the host strain caused by various 'curing' agents or conditions such as growing the host at elevated temperature, which might differentially affect replication of the plasmid (compared with the chromosome). Given the facts that many such plasmids are non-conjugative and finding the right agent/conditions for 'curing' is an arbitrary process, it is perhaps not surprising that strong proof for the widespread occurrence of plasmid-coding for catabolic genes had to await the development of adequate techniques for isolating plasmid DNA. However even this was not easy since methods developed for smaller plasmids, as for example many of the resistance plasmids, did not work for catabolic plasmids, most of which are large (80 to >300 kbp). Indeed there are many strains that degrade compounds, the catabolism of which might possibly be plasmid-encoded, for which the location of those genes has not yet been definitively demonstrated.

Although the existence of these plasmids poses interesting and wide-ranging questions concerning their role in the ecology of saprophytic populations

and in the evolution and spread of catabolism within those populations, the majority of investigations into these plasmids over the last three decades has used them simply because they are convenient vehicles for locating, isolating and investigating catabolic genes of interest. Thus, for example, the vast majority of publications on the archetypal TOL plasmid pWW0, which has been arguably one of the most studied of all catabolic plasmids², have concentrated on the two *xyl* operons, encoding the enzymes for the conversion of the primary substrates, toluene and some alkyl toluenes, to tricarboxylic acid cycle intermediates and on the two regulatory genes *xylR* and *xylS* responsible for their expression⁷⁰. Most of these studies could equally well have been carried out in the same way had the genes been cloned from the chromosome of their host, *P. putida* mt-2. A similar situation can be found if we look at other well-studied plasmids such as the two naphthalene plasmids from *P. putida* PpG7 and *P. putida* NCIMB9816, where respectively the regulation of the two operons by the single regulator protein NahR^{13, 40, 77} and the enzymology of the initial attack on naphthalene^{45, 67, 81} have been the major points of study, and similarly the phenol-catabolic plasmid from *P. putida* CF600 has been studied mainly for the regulation of the single *dmp* operon^{76, 95}. This is not to undervalue such studies since they are of vital importance in understanding the organisation and expression of these often long and biochemically interesting pathways and thereby understanding better the process of biodegradation in nature. However the plasmid-borne nature of the pathways has been a minor part of such studies. This surely has been partly responsible for the fact that more than 30 years after their discovery and in a period when the number of complete bacterial chromosomes that have been sequenced increases almost daily, the number of catabolic plasmids that have been sequenced can be counted on the fingers of one hand.

Plasmids are ubiquitous elements in genomes right across the bacterial kingdom, can transfer their genes between different strains and genera, and are probably far less subject to the rigid constraints of selection that apply to bacterial chromosomes. It is therefore not unreasonable to suppose that plasmids, in conjunction with other mobile elements such as transposons and insertion sequences, are major players, if not *the* major players, in the rapid evolution of bacteria in response to changes in the environment brought about by modern society. This appears to have been accepted in the case of the rapid evolution of antibiotic resistance in hospital and agricultural environments. If interest can be judged by the relatively minor amount of effort that has been put into sequencing of complete catabolic plasmids or, even more broadly environmental plasmids, the role of plasmids as vectors of evolutionary adaptation in the broader environment and in particular in the development of pathways capable of degrading chemicals entering the environment from the chemical industry seems to have created less impact. In contrast there is plenty of partial

information from a large number of catabolic plasmids, most of which only involves sequences of and around the genes for the pathways themselves and seldom extends into the flanking DNA.

This chapter is on *Pseudomonas* catabolic plasmids and it is outside the brief to include plasmids that have been sequenced from other genera, in particular the first completely sequenced 184 kbp catabolic plasmid pNL1 from *Sphingomonas aromaticovorans*⁷⁴ in spite of the importance of Sphingomonads as agents of biodegradation. Since very little work has been done on intergeneric transfer of catabolic plasmids, it is arguable the extent to which a particular pool of plasmids can directly transfer or be mobilised between different genera of bacteria. The gene sequences from plasmid pNL1 show that there are clear similarities between, for example, genes for hydroxylating dioxygenases in *Sphingomonas* and *Pseudomonas*, which point to a common evolutionary origin and therefore indicate that gene exchange must have taken place at some stage. However the organisation of the *Sphingomonas* catabolic genes on pNL1 is very different from that on the *Pseudomonas* plasmids and shows little of their organisation into discrete operons. It seems likely that pNL1, and presumably other *Sphingomonas* plasmids, constitute a separate plasmid pool, albeit one between which gene exchange has taken place in the past, probably involving transposition and broad host-range plasmids as intermediate genetic vehicles for the genes.

2. THE TOL PLASMID pWW0 FROM *P. PUTIDA* mt-2

The archetypal TOL plasmid pWW0 originates in *P. putida* mt-2, a strain originally isolated in Japan in the 1950s⁵⁵. Strain mt-2 first came into prominence as the source of the first catechol 2,3-dioxygenase (metapyrocatechase) to be purified and studied⁶³. The plasmid location of catabolic genes was first demonstrated in the early 1970s (as before) and it was subsequently shown to encode the ability to convert toluene, and 3- and 4-methyltoluenes (*m*- and *p*-xylenes) to central metabolites¹⁰³. In spite of a considerable body of work concentrating on its catabolic genes and their regulation, that included several papers on their sequence, the complete sequence of the plasmid was not published until 2002³⁰ (Figure 1; NC 003350), although the main elements of its structure were already known in broad outline. The catabolic genes are organised in two operons, the 'upper pathway' operon *xyIUWCMABN* and the 'meta pathway' operon *xyWXYZLTEGFJQKIH*², which are transcribed in the same direction, with a divergently transcribed pair of regulator genes close to the 3' end of the latter⁷⁰. The two operons are separated by 11 kbp of non-catabolic DNA, containing 14 open reading frames (ORFs), which apart from two

transposase-like genes, are of unknown function. The entire catabolic region is clustered on 37.7 kbp, about a third of the entire plasmid, between two identical direct repeats of 1,275 bp. These have the sequence characteristics of an insertion sequence with an internal transposase (*tnpA*)-like ORF, terminal inverted repeats of 12 bp, and have been termed *IS1246*⁷¹. *IS1246* has not been demonstrated to transpose, nor has the region spanned by the two copies been shown to act as a composite transposon but the two direct repeats of *IS1246* do have a phenotypic consequence for the plasmid in providing considerable areas of homology between which reciprocal recombination can occur. This can result in deletion of 39 kbp, removing the entire catabolic region and forming a 78-kbp cryptic plasmid pWW0-8 with no catabolic phenotype and

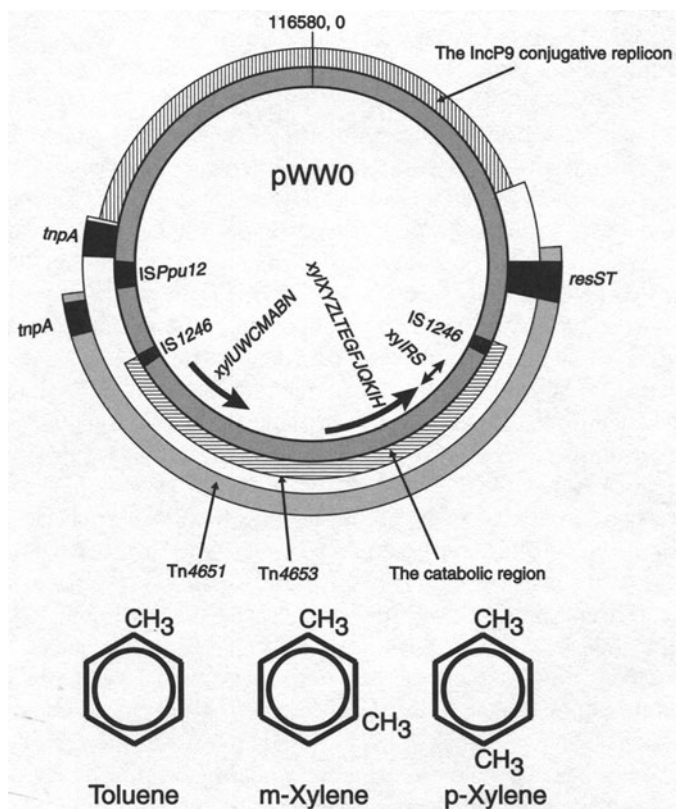


Figure 1. Map of TOL plasmid pWW0 and its catabolic substrates. Specific IS elements and transposase genes described in the text are highlighted in black. The location of the *xyl* catabolic genes and their direction of transcription are denoted by the arrows.

only one copy of IS1246³. This loss of phenotype can be selected for by growth of pWW0⁺ cells on benzoate when faster growth by the chromosomal β -ketoadipate pathway amplifies any cells carrying spontaneously deleted plasmids¹⁰¹. It is likely that the majority of such 'cured' strains arise from *P. putida* mt-2 by this deletion rather than by plasmid loss¹⁰⁰.

The plasmid has three different elements, which have been shown to transpose. Two of these were first reported by Tsuda and Iino^{84, 85} and are large nested transposons, both of which span the catabolic region. Tn4651 is the smaller of these at 56 kbp and is itself encompassed within the larger 78-kbp Tn4653. Both have characteristics of class II transposons and can transpose independently⁸⁷, but have a degree of interdependence since although both have separate large *tnpA* genes, they share common resolution genes *tnpI*, *tnpS* and *res*⁸⁷. If the catabolic region is deleted, as in pWW0-8, both of these large transposons still function but as cryptic transposons and are called Tn4652 (Tn4651) and Tn4654 (Tn4653).

Additionally a third 3372-bp insertion sequence IS*Ppu12* has been identified on pWW0⁹⁷. This appears to have played some part in the past laboratory history of pWW0 since a number of catabolically defective strains had been reported in which a similarly sized element had appeared within or close to the *xyl* genes. Indeed its isolation and sequencing were independently carried out on a fragment from a derivative of pWW0, defective in meta pathway expression, which had been transferred into a chlorobenzoate-degrading *Pseudomonas* sp. B13. Only subsequently was the same sequence found to be located on wild-type pWW0 in a different position. IS*Ppu12* is highly promiscuous and was found in multiple copies on the chromosome of *Pseudomonas* B13 into which pWW0 has been transferred and in four copies on a copy of pWW0 isolated from a B13 mutant. Yet remarkably there are no copies of it in the chromosome of its normal host *P. putida* KT2440 (plasmid-free *P. putida* mt-2)^{59, 72, 97}.

The plasmid functions of pWW0 (replication, stable inheritance and conjugation) are all clustered within 46 kbp in what has been termed the IncP-9 core of the plasmid. On the basis of this Greated *et al.*³⁰ have proposed that the current plasmid evolved from this 46-kbp plasmid by the sequential accretion of transposons starting with an 11.3-kbp precursor of the large transposon (Tn4653), followed by insertion into that of the 14.4-kbp precursor of Tn4651 followed by the later insertions of IS*Ppu12* and the 39-kbp catabolic region between the two copies of IS1246, perhaps as a composite transposon (Figure 2). Further examination of the region at the termini of the catabolic region suggests that this latter may have occurred by a more complex set of events (see below) but the main deduction that pWW0 has evolved through successive recombination/transposition events to give its present complex structure is undoubtedly correct.

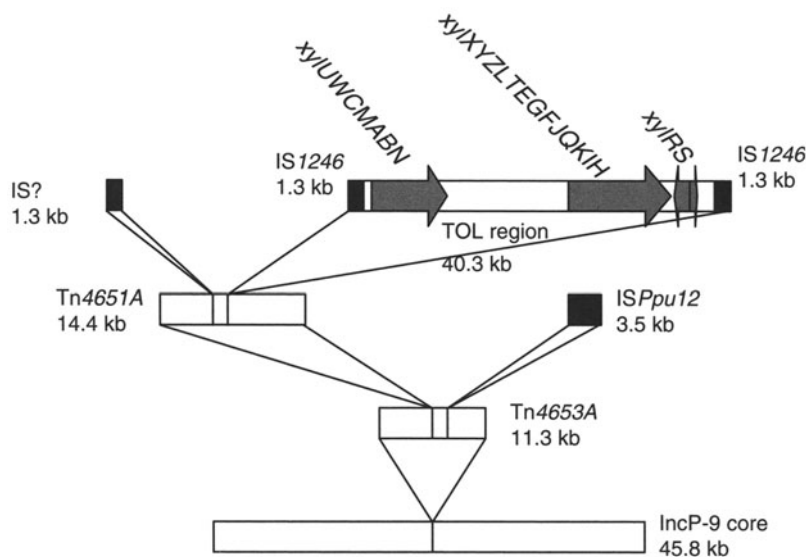


Figure 2. Possible sequence of recombination events leading from a basic IncP-9 core to the present structure of pWW0. IS? represents a possible insertion sequence suggested by the sequence analysis but which has not been investigated experimentally. The catabolic operons and their directions of transcription are denoted by grey arrows and relevant insertion sequences in black. The figure is not to scale. Adapted from ref. [30].

The presence of three functional transposable elements on pWW0 plus the high frequency of conjugational transfer of the plasmid indicates the possibility that the complete unit can still participate in the spread of its DNA either as a whole or in part and thus be a factor in microbial evolution. Examination in detail of the entire structure of the plasmid also suggests that in its past history it has participated in a whole series of other recombination/transposition events, the residues of which are still maintained although not necessarily in a functional form any more. For example, including the functional transposases associated with Tn4651, Tn4653 and ISPpu12 and the *tnpA*-like genes on the two copies of IS1246, there are a total of 12 ORFs, the closest genes to which are transposases. There are also a number of genes that are clearly related to metal resistance (e.g., against mercury), which are distributed around the non-replicon region, although comparison of wild-type *P. putida* mt-2 with plasmid-free derivatives have given no indication of any metal resistance conferred by the plasmid. It seems therefore likely that many of these genes are non-functional residues left over from a past history of continual structural modification but which have been retained because they

present a negligible selective disadvantage to the symbiotic lifestyle of the plasmid.

Since the first identification of pWW0, many other TOL plasmids have been identified that carry the same catabolic phenotype but which are of different sizes, many of them considerably larger^{47, 78, 101}, and many of which carry gene duplications of entire catabolic operons or parts of catabolic operons^{64, 65, 78}. Much as the comparative sequencing of different enteric bacterial chromosomes has led to an understanding of the ways the housekeeping genes of bacteria evolve, it seems likely that a similar comparison of a subset of such plasmids would establish a wealth of information about the role of plasmids in conjunction with transposons and other mobile genetic elements in the past and present history of bacterial evolution in the fast track.

3. THE NAPHTHALENE PLASMID pDTG1 FROM *P. PUTIDA* NCIB 9816-4

Just as toluene and phenol are the model systems for monocyclic aromatic hydrocarbon degradation the two-ring compound naphthalene has long been used as a model system for polycyclic aromatic hydrocarbon degradation. As mentioned above, in toluene degradation there is a methyl-group activating upper catabolic pathway and a ring-oxidizing and ring cleavage lower catabolic pathway. The fused two-ring naphthalene catabolic pathway poses a different metabolic challenge: how to sequentially metabolize each ring in a productive fashion. Since many of the naphthalene catabolic pathways are capable of metabolizing the three-ring compound phenanthrene as well, an additional challenge is to evolve a catabolic pathway able to sequentially remove each of the three rings of phenanthrene. Naphthalene and phenanthrene metabolism must be able to proceed simultaneously, as these compounds are often found together in nature. Following the model of the TOL plasmid above, one would expect to find that a NAH plasmid-encoded catabolic pathway would have an upper pathway for metabolism of the first ring and a lower ring-cleavage pathway for the remaining aromatic ring. In the case of the three-ring compound phenanthrene, metabolism would proceed by passing the compound through the upper part of the pathway twice before the final aromatic ring is metabolized.

This catabolic pathway for naphthalene degradation has been studied as early as 1964 by Davies and Evans¹⁷ and elegant investigations 30 years later by Eaton and Chapman²⁴ have provided more detail on the individual enzymatic steps following ring cleavage of 1,2-dihydroxynaphthalene. Naphthalene degradation is initiated by the introduction of both atoms of molecular oxygen

into the aromatic nucleus through the action of naphthalene dioxygenase²⁵. This multicomponent enzyme system consists of a reductase³⁵, a ferredoxin³⁶, and an iron sulfur protein²⁶ and catalyzes the oxidation of naphthalene to *cis*-(1R,2S)-dihydroxy-1,2-dihydronaphthalene (*cis*-naphthalene dihydrodiol). Subsequent enzymatic reactions by *cis*-naphthalene dihydrodiol dehydrogenase and 1,2-dihydroxynaphthalene dioxygenase result in the formation of 2-hydroxy-4-(2'-oxo-3,5-cyclohexadienyl)-buta-2,4-dienoate²⁴. Further reactions lead to the formation of salicylate, which in NAH-plasmid strains is metabolised through catechol to TCA cycle intermediates and which in other organisms is metabolised through gentisate²⁹. The enzymes involved in naphthalene degradation also have the ability to completely degrade the three-ring compound phenanthrene and partially degrade the three-ring compound anthracene at least through removal of one of the rings with the formation of pyruvate and hydroxynaphthoate^{27, 52, 105, 112}.

As mentioned above, the first published report of a naphthalene catabolic plasmid was in 1973 by Dunn and Gunsalus²². These authors observed conjugative transfer of the ability to grow on naphthalene from *P. putida* strain PpG7 to other strains as well as loss of the naphthalene positive phenotype in the presence of the curing agent mitomycin C. This general class of plasmids was labeled NAH and the specific plasmid in strain G7 named NAH7. Subsequent DNA isolation and transformation experiments physically confirmed the role of plasmid NAH7 in naphthalene degradation⁴⁴. Restriction mapping, transposon mutagenesis, and subcloning experiments all led to a physical and functional map of NAH7^{106, 108}. As expected, two operonic gene organizations were discovered, one encoding the metabolism of naphthalene to salicylate and one encoding the metabolism of salicylate to pyruvate and acetaldehyde. The two operons are adjacent to one another with a several kilobase pair gap between them and make up approximately one third of the total >80 kbp size of the plasmid. Both operons are induced by salicylate, through the action of NahR, encoded by a gene adjacent to, and transcribed in the opposite direction from, the lower pathway operon. Many other NAH plasmids were identified in the next 10 years, summarised in an excellent review by Yen and Serdar¹⁰⁷.

Although the earliest genetic studies were carried out with plasmid NAH7, the best-studied naphthalene-utilising *Pseudomonas* strain from a metabolic standpoint is NCIB 9816. This strain was originally isolated from soil by Davies and Evans in 1964¹⁷. However, some ambiguity exists in the published literature regarding the strain, suggesting that different variants of NCIB 9816 have arisen over time and in passage through different laboratories (summarised in Yen and Serdar¹⁰⁷). This suggests that plasmid-encoded naphthalene metabolism is subject to continuing evolution and genetic flux. Williams *et al.*⁹⁶ reported that growth of NCIB 9816 on naphthalene induced

1,2-dihydroxynaphthalene oxygenase and the catechol meta-cleavage enzyme, catechol 2,3-oxygenase. Growth on salicylate induced the catechol ortho-cleavage enzyme, catechol 1,2-oxygenase. This strain was designated 9816-1. In a second variant 9816-2, Barnsley⁴ saw constitutive catechol meta-cleavage enzyme production, with naphthalene and salicylate inducing the synthesis of the naphthalene upper pathway enzymes as well as salicylate hydroxylase and catechol ortho-cleavage enzymes. Strain 9816-3 grown on salicylate and naphthalene induced catechol ortho-cleavage enzymes, while catechol meta-cleavage enzymes were constitutively expressed at low levels⁴. Strain 9816-4 from the Gibson laboratory has induction characteristics similar to 9816-3, however, there are differences in the number and size of plasmids found in these two strains⁷⁹.

The plasmid content of NCIB 9816-4 was initially examined by Serdar and Gibson⁷⁹. A single 83 kbp plasmid was detected and designated pDTG1. Deletion mutants of pDTG1 were readily constructed following exposure to halogenated naphthalenes or salicylates and identified restriction fragments containing the upper and lower catabolic pathway genes⁷⁹. An *EcoRI* digest of the three variants of the NCIB 9816 plasmid, pDTG1^{79, 80}, pWW60-1 from *P. utida* NCIB 9816-3^{11, 12}, and NAH2 from *P. putida* NCIB 9816-2¹⁶, in comparison to NAH7 from *P. putida* G7^{80, 106}, showed that these three plasmids are not identical, and are in fact quite different. However, comparison of the various naphthalene catabolic gene sequences known for both NAH7 and pDTG1 shows greater than a 90% identity. For example, the genes encoding naphthalene dioxygenase from NAH7 and pDTG1 are greater than 90% identical but the identity rapidly diverges outside of the catabolic operon⁸⁰. The genes for the initial steps in the degradation of naphthalene have been cloned and sequenced from many different *Pseudomonas* strains. In almost all cases the genes are present on plasmids in the original strains. Interestingly, even though these plasmids are properly classified as NAH plasmids, the initial substrate on which some of these different *Pseudomonas* strains were isolated includes phenanthrene⁴⁶ and dibenzothiophene¹⁸. In some *Pseudomonas* strains the NAH plasmid-like genes are found in the chromosome. The genes for naphthalene degradation in *P. stutzeri* AN10 are one such example^{8, 9}. This is not surprising as a 37.5-kbp region of NAH7 is capable of transposition⁸⁶. However, in the case of NAH7 the 'transposon', designated Tn4655, does not encode a functional transposase but does encode a functional resolvase. The *nah* gene region can be made to transpose upon supplying a Tn1721-class transposase *in trans*.

The complete nucleotide sequence of plasmid pDTG1 has been determined (Figure 3; AF491307). The length of the plasmid is just over 83-kbp, precisely as predicted by the restriction endonuclease digests. Analysis of the NAH plasmid pDTG1 sequence reveals significant identity at the nucleotide

sequence level to the TOL plasmid pWW0. The ‘backbone’ of both plasmids is a homologous region encoding for replication and conjugation. In the case of pDTG1 the upper and lower *nah* operons have been added to the backbone. The upper pathway *nah* operon contains the *nahAaAbAcAdBFCQED* genes encoding for the conversion of naphthalene to salicylate (and phenanthrene to 1-hydroxy-2-naphthoate). The lower pathway *nah* operon contains the *nahGTHINLOMKJY* genes encoding the meta-ring fission pathway and chemotaxis. The *nahR* gene is adjacent to *nahG* but transcribed in the opposite direction. Interestingly, there is an IS5 family transposable element inserted between the *nahG* (encoding salicylate hydroxylase) and *nahT* genes. This explains the observation described above that NCIB 9816-4 degrades naphthalene through catechol and the ortho-cleavage pathway. There must be a polar effect on the *nahH* gene encoding catechol 2,3-dioxygenase. Since NCIB 9816-4 is chemoattracted to naphthalene³¹, presumably through the action of the *nahY* gene product³², there must be an internal promoter in this lower ‘operon’ after the insertion point.

The upper and lower *nah* operons are separated by over 16-kbp of DNA. Transcription of each operon proceeds toward the other. Interestingly, even though plasmid NAH7 has not yet been sequenced, the published restriction

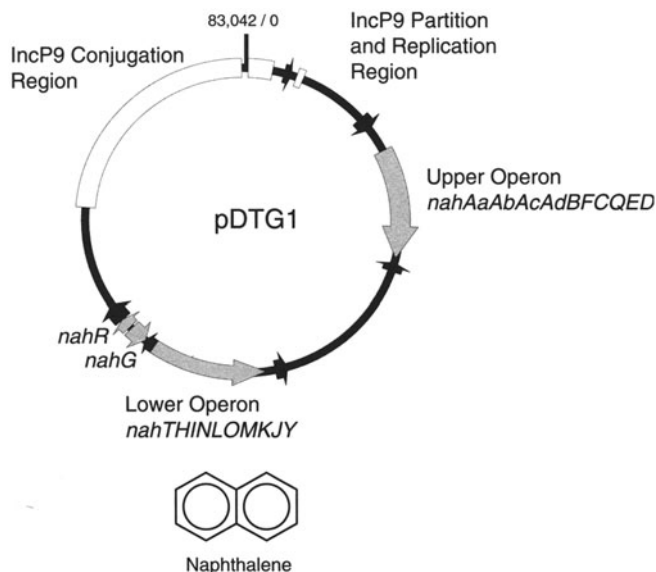


Figure 3. Map of NAH plasmid pDTG1 and its catabolic substrate. The catabolic genes are denoted by grey arrows and genes or pseudogenes related to known transposition genes are shown as black arrows.

map of the NAH7 *nah* genes^{106, 108} indicates that the upper and lower operons are transcribed in the same direction and that the two operons are separated by less than 5-kbp. The pDTG1 sequence only contains one potential match for the 38 bp inverted repeats described by Tsuda and Iino⁸⁶ for Tn4655 containing the *nah* genes from NAH7. This single match is midway between the upper and lower *nah* operons and thus cannot function as in NAH7 to allow transposition of the *nah* genes. Although a match to the 38 base pair inverted repeat can be found between pDTG1 and NAH7, the DNA sequences surrounding the 38 base pair inverted repeat do not match between the two plasmids. This 38 base pair inverted repeat can however, be found flanking the two *xyl* gene operons in pWW0, approximately 70 kb apart. This analysis leads to the hypothesis that the NAH7 and pDTG1 plasmids may have arisen separately from each other, through different recombinational or transpositional events, and not simply be evolutionary variants of each other, derived from a common ancestor.

The sequence determination of pDTG1 has permitted at least a partial reexamination of some of the variation between plasmids from *P. putida* NCIB 9816. Much of the earlier strain differences concerned whether naphthalene utilisation was via the meta- or ortho-cleavage route for catechol catabolism. In the strain used in Bangor, we demonstrated that it used ortho-cleavage of catechol but that selection for growth on 2-methylnaphthalene (which can only be dissimilated by the meta pathway) resulted in deletion of DNA upstream of *nahH* and consequent normal expression of the meta pathway¹¹. We have subsequently returned to sequence this part of the pWW60-1 (unpublished results) and found that, from *nahT* through *nahH*, it is identical to pDTG1 and both carry what appears to be a small insertion sequence in the intergenic region. In the two deletion mutants of pWW60-1, which express the meta pathway, the IS element has been deleted. This identity of pDTG1 and pWW60-1 suggests a very close relationship and that both have a recent common ancestor in which expression of the meta pathway had become blocked by the acquisition of this IS element within the meta pathway genes causing a polar mutation and diverting the catabolism of the catechol formed from salicylate through the chromosomal β -ketoadipate (ortho) pathway.

4. THE ATRAZINE PLASMID pADP-1 FROM *PSEUDOMONAS* SP. ADP

The recent determination of the complete nucleotide sequence of the atrazine catabolic plasmid pADP-1 from *Pseudomonas* ADP (Figure 4; U66917) provides additional insights into how plasmids evolve carrying genes for the catabolism of compounds recently added to the biosphere. It is

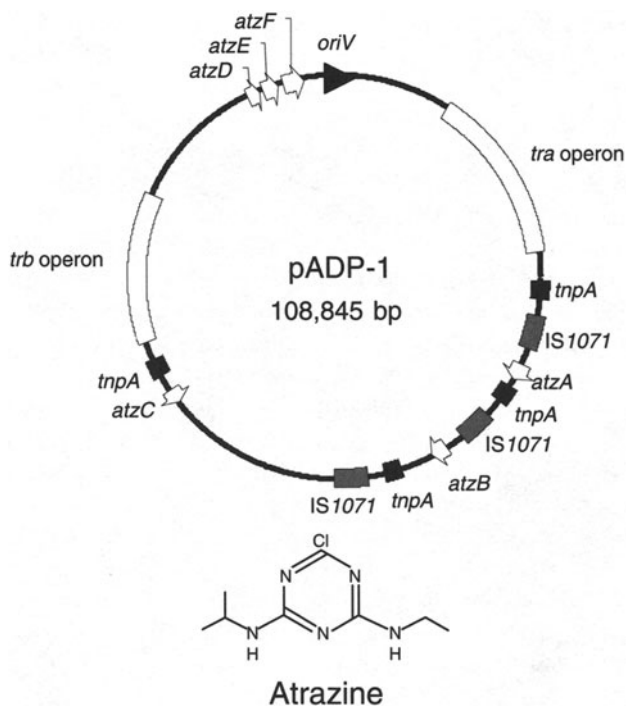


Figure 4. Map of atrazine plasmid pADP-1 and its catabolic substrate. Transposition related genes are in black, insertion sequences in grey and the catabolic genes are hatched. Adapted from ref. [51].

estimated that half a million metric tons of the *s*-triazine ring pesticide atrazine has been applied to agricultural land globally since 1960. This massive usage, coupled with the fact that atrazine is considered only moderately biodegradable, has resulted in its accumulation in environmental aquatic systems. Furthermore, as far as we are aware, no natural *s*-triazine ring products are known in biology so, distinct from the TOL and naphthalene plasmids, it provides an example of a plasmid vector encoding a recently evolved pathway.

Pseudomonas sp. ADP was isolated from a herbicide spill site and was found to be capable of metabolizing atrazine in liquid cultures, agar plates and solids at relatively high concentrations. It was able to completely mineralize the triazine ring, using the ammonia released as its sole nitrogen source⁵⁰.

Prior to the publication of the complete nucleotide sequence of pADP-1 from *Pseudomonas* ADP, only the first three steps of the atrazine pathway had been described in this bacterium. The steps, encoded by genes *atzA*, *atzB* and *atzC*, transform atrazine to cyanuric acid^{10, 20, 21, 75}. The first enzyme, AtzA,

catalyses the hydrolytic dechlorination of atrazine, yielding hydroxyatrazine. This is converted by the second enzyme, AtzB, to N-isopropylammelide, and the third enzyme, AtzC, N-isopropylammelide isopropylaminohydrolase, stoichiometrically produces cyanuric acid and N-isopropylamine. However, the complete sequence of the plasmid allowed the researchers to identify the entire catabolic pathway for atrazine degradation in this strain.

The pADP-1 catabolic plasmid was found to be 108,845-nucleotide bases in length (Figure 4). One half of the plasmid has the classical IncP β backbone similar to that of the archetype IncP β plasmid pR751. It contains a total of 104 ORFs, with 15% of them predicted to be involved in catabolism, and 15% predicted to be involved in transposition. The remaining ORFs were predicted to be involved in general metabolism, in plasmid maintenance, transfer and replication, as transcriptional regulators, or as putative transporters⁵¹. Sequence analysis suggested that the genes that code for the enzymes which catalyse the remaining steps of the atrazine pathway (*atzD*, *atzE* and *atzF*) were also present on pADP-1 and this was confirmed by biochemical analysis. AtzD hydrolyzes cyanuric acid to biuret, AtzE converts biuret to allophanate, and AtzF converts allophanate to CO₂ and NH₃, thus revealing that all of the genes of the atrazine pathway in *Pseudomonas* ADP are encoded on pADP-1.

Whereas the *atzA*, *atzB* and *atzC* genes were found to be dispersed at positions *c.* 35, 45, and 71 kbp on pADP-1 respectively, *atzDEF* are adjacent between positions 101 to 106 kbp, and are cotranscribed. Furthermore, divergently transcribed from *atzDEF* is a predicted regulator gene similar to the LysR family of transcriptional regulators. Analysis showed that the regulator-like gene, and the *atzDEF* genes have similar G+C contents (59–61%), suggesting that these four genes together constitute an operon plus regulator gene.

Analysis of the G+C content of the *atzA*, *-B* and *-C* genes established that *atzC* has a lower G+C content (44%) than those of *atzA* (58%) and *atzB* (61%). This suggests that *atzC* was acquired from an organism with vastly different G+C content from the source of *atzA* and *atzB*.

There is no sequence evidence for regulatory elements upstream of *atzA*, *-B* or *-C* genes, and the evidence indicates that *atzA* and *atzB* genes are transcribed in the absence of atrazine, thus concluding that *atzA*, *-B* and *-C* are constitutively expressed. This absence of a regulatory mechanism for the expression of these catabolic genes also suggests that they were recently acquired by *Pseudomonas* ADP and efficient regulation of expression has not yet been selected for (compare below).

The DNA regions surrounding each of the *atzA*, *-B* and *-C* genes were found to contain complete copies of a transposase similar to TnpA from IS1071. Each one formed structures resembling nested catabolic transposons.

Since the DNA sequences of the insertion elements flanking the *atzA*, *atzB* and *atzC* genes are nearly identical to known active *IS1071* elements, it is probable that atrazine catabolic genes have been recruited by independent transposition or homologous recombination events from other replicons and ultimately other bacteria. This was inferred in an earlier study which showed that five geographically distinct atrazine-degrading bacteria, including members of the genera *Alcaligenes*, *Ralstonia* and *Agrobacterium* contain *atzA*, *atzB* and *atzC* genes that are homologous to those in pADP-1¹⁹. In the study, DNA sequence of up to 600 bp from *atzA*, *atzB* and *atzC* was PCR amplified from members of these genera and compared to that of the same genes from pADP-1. Identities of these *atz* genes were >99% to those of pADP-1, indicating that globally distributed atrazine-catabolic genes are highly conserved in diverse genera of bacteria.

pADP-1 is a self-transmissible plasmid capable of being transferred to *Escherichia coli* strains at a high frequency. This adds further evidence for a potential molecular mechanism for the dispersion of the *atzABC* genes between soil bacteria^{19, 20}.

These findings provide a window onto the apparent recent evolution of catabolic pathways in nutritionally diverse soil bacteria. Furthermore, comparisons of the complete nucleotide sequences of other atrazine catabolic plasmids will show these modules, or blocks of genes as footprints of lateral gene transfer.

5. THE CARBAZOLE/DIOXIN PLASMID pCAR1 FROM *P. RESINOVORANS* STRAIN CA10

This 199,035-bp circular plasmid (Figure 5; AB 088420) contains 190 ORFs and encodes gene clusters for the degradation of carbazole (*carAaAaAbBaBbCac*AdD**FE*) and anthranilate (2-aminobenzoate) 1,2-dioxygenase (*antABC*)⁴⁹; the asterisks denote ORFs with no known function. Carbazole is a natural N-containing heteroaromatic compound found in tars and shale oils and is initially converted in an angular dioxygenation to 1-hydro-1,9a-dihydroxycarbazole⁶² by a three-component dioxygenase system (CARDO; CarAaAcAd), the terminal oxygenase of which is a trimer of a single monomer (CarAa)₃⁵⁷. Its structure therefore differs from naphthalene 1,2-dioxygenase (NDO), which is a hexamer of two subunits, (NahAc)₃(NahAd)₃⁴⁵, although it has a broad specificity and will catalyze the same conversion of naphthalene to the same dihydrodiol as does NDO⁵⁷. Further metabolism of this by the enzymes of the *car* cluster yields anthranilate, which is subsequently converted to catechol by AntABC, a three-component dioxygenase system in the same family as benzoate 1,2-dioxygenase⁵⁸, and catechol is further metabolised via the chromosomally encoded β -ketoadipate

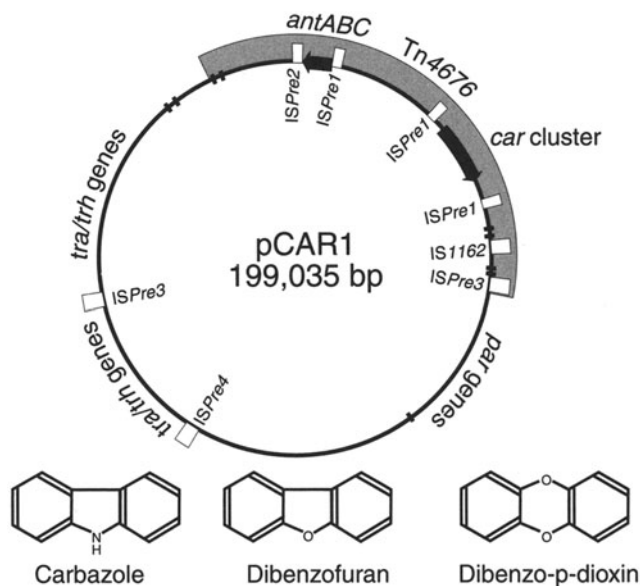


Figure 5. Map of carbazole plasmid pCAR1 and its catabolic substrates. The eight IS elements described in the text are shown as white boxes and other genes with similarities to known transposition or integration functions are shown as black dashes. The catabolic genes are denoted by black arrows. Adapted from ref. [49].

pathway. Presence of pCAR1 enables the host to use carbazole as the sole source of carbon, nitrogen and energy but also to degrade dibenzofuran, the O-homologue of carbazole, and dibenzo-*p*-dioxin and some of its chlorinated derivatives³⁴ (Figure 5).

In many ways the structure of pCAR1 bears some striking resemblances to pWW0. The two catabolic gene clusters are located on a large 72.8-kbp element, which bears all the hallmarks of a transposon, has been named Tn4676, and has recently been demonstrated to transpose (H. Nojiri, personal communication). Not only does this element contain genes with 80–90% identity to *tnpA*, *tnpC*, *tnpR* and *tnpST* of the TOL transposons Tn4651 and Tn4653, it also contains six sequences that show >67% identity with the 46-bp IR repeat of Tn4651, and the outer copies of these are spanned by a 5-bp direct repeat characteristic of the past insertion of a transposon. In addition to this putative catabolic transposon, pCAR1 also carries six small insertion sequences. Two of these were earlier identified as they are closely associated with the two catabolic gene clusters⁶¹. The *car* cluster is bounded by two copies of ISPre1 and the *ant* genes by one copy of ISPre1 and one of ISPre2. The copy of ISPre1, adjacent to *carAa*, appears to be the result of an internal imprecise transposition

of the copy adjacent to *antA* since it has carried with it a copy of the 5' end of *antA*. This implies that *ISPre1* is still an actively transposable element but also that the apparent similarity in structure of the *car* gene cluster to a composite transposon (i.e., flanked by two IS elements) is not a reflection the way the cluster was acquired by pCAR1. However it is possible that *antABC* was acquired as a composite transposon and also that both the *ant* and the *car* clusters in their present configurations on pCAR1 could be vehicles for further transposition. Of the additional IS elements identified during the complete sequencing⁴⁹, two are members of the *IS21* family, one being virtually identical to *IS1162*, previously reported in a *P. fluorescens* strain, and one shows similarities to *IS1491* from a *P. alcaligenes* and has been named *ISPre4*. Additionally there are two identical copies of *ISPre3*, which resembles an *E. coli* IS element *ISEc8* but which contains an additional ORF not present in *ISEc8*. Although none of these IS on pCAR1 have yet been shown to transpose, they are all located between credible IRs and are highly likely to be active still. Six of them are located with the larger *Tn4676*. Like pWW0 the complete annotation of pCAR1 reveals a remarkably high frequency of transposition-related genes, and within the total of 190 ORFs, 24 show homologies with known functional genes in the databases.

A further point of similarity with both pWW0 and pADP1 is a degree of spatial heterogeneity in the composition of the DNA around the plasmid. In particular the two catabolic gene clusters and their flanking DNA show strikingly dissimilar composition, the *ant* genes and its flanking DNA being GC-rich (64%) whereas the region containing the *car* genes were, for *Pseudomonas* genes, relatively GC-poor at 51%.

The mosaic structure (Figure 6) shows that this plasmid, like the other catabolic plasmids, bears strong evidence of a past history of multiple

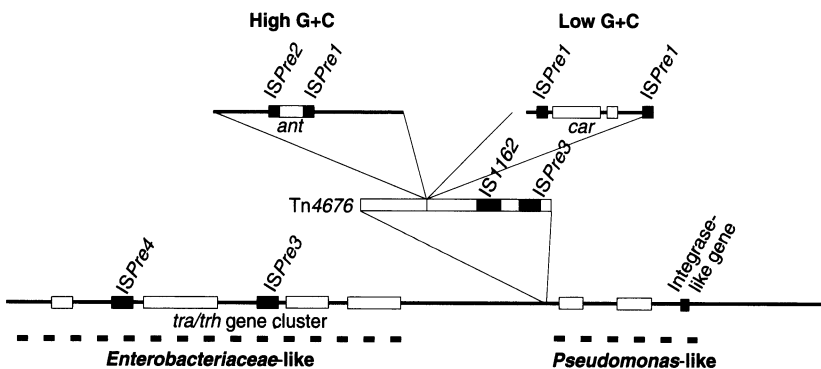


Figure 6. Mosaic nature of pCAR1 structure. The circular structure has been linearized to indicate the various different elements that make up the structure. Figure provided by H. Nojima.

recombination events, and has undoubtedly been assembled from a replicon that has acquired additional elements, in particular those that enable it to endow its hosts with the ability to degrade both naturally occurring compounds but additionally man-made compounds with potential toxicity and pollution hazards (chlorinated dioxins).

6. PLASMIDS, PATHWAYS AND EVOLUTION

The unique feature of *Pseudomonas* catabolic plasmids is that they carry genes for the complete catabolism of a wide variety of the more unusual growth substrates. In many cases the substrates for the plasmid pathways, for example, naphthalene, phenol and toluene are compounds that have probably been around in the environment for considerable periods of time and therefore the pathways, although not necessarily the plasmids, represent a stable and successful result of past evolution. However in other cases, for example, atrazine and nitrotoluenes, the compounds are almost certainly very recent additions to the environment and the existence of complete pathways for their degradation must also be of recent origin. The fact that the genes for such pathways are invariably found on plasmids and/or transposable elements indicates that the ability of such elements to move between strains must be a major factor in the evolution of such novel pathways. On reflection this may be stating no more than the obvious since, without gene transfer, such evolution would require all the necessary genetic information to come from within any one bacterium, which would severely limit the degree of novelty that could be achieved. However, given the fact that the saprophytic bacterial population as a whole contains a vast and unknown number of genera, species and strains (culturable and non-culturable), all of that have evolved to exist in a highly competitive environment with an unpredictable supply and composition of nutrients, it is not surprising that any process that can effect even a moderate degree of gene reassortment between different hosts will be evolutionarily much more powerful.

The concept of modular pathway construction and evolution from preexisting genetic elements has been proposed and discussed in a number of publications^{88, 89, 92, 99} and the well-characterised plasmid-borne pathways provide several examples where such a reassortment of genes must have taken place to give the gene structure seen. Within *Pseudomonas* there are the meta-cleavage (lower) pathway operons found on TOL, naphthalene and phenol plasmids. In these a stable substrate (benzoate/methyl benzoates, salicylate or phenol respectively) is converted first to catechol and then subsequently via the meta-ring cleavage pathway to pyruvate and acetyl CoA. In all cases the genes in *Pseudomonas* for the pathway from catechol down are highly

homologous and in an identical gene order (Figure 7). It is in the genes for the first metabolic reactions, which are also the upstream genes in the operons, that they differ completely. TOL plasmids have *xylXYZL* (for benzoate dioxygenase and benzoate diol dehydrogenase³⁷, naphthalene plasmids have *nagG* (for salicylate hydroxylase¹⁰⁴ and phenol plasmid pV1150 has *dmpKLMNOP* (for the six-component phenol hydroxylase⁶⁹; none of these share any similarity to each other. The rational scenario for the evolution of these operons is that they have been formed by fusions between a common element (for catechol catabolism) and a different initial gene or gene cluster (Figure 7). This modularity of construction is reinforced since homologues of the *xylXYZL* cluster of TOL plasmids are found independently and in a functionally different situation as *benABCD* used for the initial steps in the dissimilation by the β -ketoadipate pathway in both *Pseudomonas*⁴³ and also in *Acinetobacter*⁵⁸, and in both cases are chromosomally located. Another related cluster of genes that appear to have been utilised as module or cassette encode the hydratase, acetaldehyde dehydrogenase and aldolase for the conversion of 2-oxo-4-pentenoate to pyruvate and acetyl-CoA, found centrally within the catechol meta-cleavage operons (*xylJQK* in the TOL plasmids). In the catabolism of biphenyl³⁹, *p*-cumate²³ and cumene³³, 2-oxo-4-pentenoate is a metabolite of pathways that differ from the 'classical' meta pathway sequence. Homologues of *xylJQK* are present in these pathways in the same gene order as in the meta pathway operons but with different surrounding genes.

A further example can be found on the TOL plasmid, where a complete pathway appears to have been assembled in a modular fashion. Whereas the

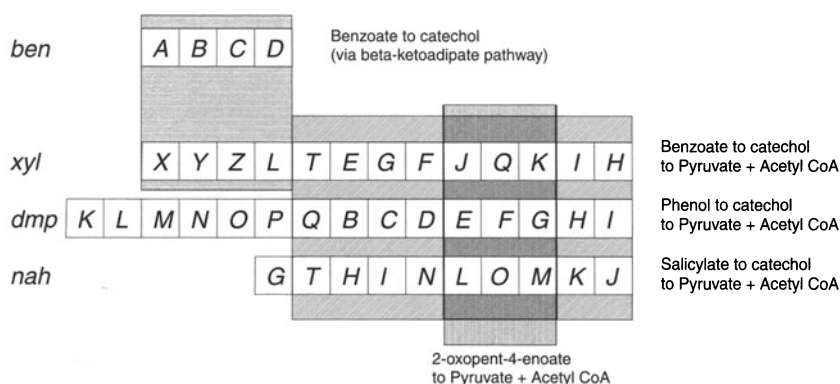


Figure 7. Modular structure of pathways for aromatic catabolism. Each of the three 'modules' highlighted by the hatched boxes (benzoate to catechol, catechol to pyruvate + acetyl-CoA, and 2-oxopent-4-enoate to pyruvate + acetyl-CoA) turns up as a homologous and identically organized cluster of genes but in different genetic contexts as stated in the text.

meta pathway operon has a mean G+C content around 62%, typical of *Pseudomonas* genes, the genes of the upper pathway operon (*xylUWCMABN*), determining toluene conversion to benzoate, all have a significantly lower G+C content of around 50% suggestive of a separate origin within a low G+C genus. This particular module, as defined by highly homologous genes in the same order, has also been found on the catabolic plasmid of a 4-nitrotoluene degrading strain *P. putida* TW3, where the identical reactions are catalysed (4-nitrotoluene to 4-nitrobenzoate) but where there is no metabolically associated meta pathway operon and the 4-nitrobenzoate is channelled down an entirely different route, specific for nitrosubstituted compounds⁴¹. In the plasmid of TW3 this operon also has a similarly low G+C content compared with the genes for nitrobenzoate catabolism⁴².

The modular construction of a 'mature' pathway is apparent in the catabolism of naphthalene reported in both a *Ralstonia* strain U2^{29, 109} and in a *Comamonas* GZ42¹¹¹, but studied in detail only in the former. The naphthalene catabolic (*nag*) genes of strain U2 encode the conversion of naphthalene to salicylate as described above for the plasmids of *P. putida* strains PpG1 and NCIB 9816. The relevant enzymes and genes for these initial steps (*nagAaAbAcAdBFCQED*) are, by the criterion of homology and an identical order, a common module shared with the 'classical' *nah* pathway of pDTG1. The *nag* pathway differs from the *nah* pathway in two important respects: (a) the salicylate formed is metabolised via gentisate (2,5-dihydroxybenzoate) instead of via catechol and the meta-cleavage pathway, and the genes for the metabolism of gentisate (*nagJKLMN*) are part of the same large operon as the genes for the initial enzymes and (b) the genes for a monooxygenase (salicylate 5-hydroxylase) for conversion of salicylate to gentisate (*nagGH*) are inserted between the two genes for electron transport proteins of the naphthalene dioxygenase system (NDO) *nagAa* and *nagAb*.

Whereas the above examples suggest that assembly of gene 'modules' has been an important element in past pathway evolution, a number of examples are present in the literature, which suggest that the same mechanisms are functional in the recent evolution of 'xenobiotic' degradative pathways and, in all cases, plasmids appear to be implicated. In the atrazine plasmid, discussed above, the three genes of the novel pathway (*atzA*, *atzB*, *atzC*) have clearly been assembled onto the same vector from different sources. However they are unregulated suggesting that regulation is a secondary process that follows initial pathway evolution. A similar situation is also found in strains growing on nitrobenzene⁴⁸ and nitrotoluene^{66, 82} in which the initial attack on the nitroaromatic ring is by a dioxygenase. The four genes for the nitrobenzene/nitrotoluene dioxygenases are clearly related to the NDO genes of *Ralstonia* U2 by both closer homology than to the classical *nah* NDO genes and because they also retain incomplete pseudogenes homologous to *nagGH* between the

Aa and *Ab* genes^{48, 66, 82}. Interestingly, like *atzABC* there appears to be very poor regulation of expression of the dioxygenase genes suggesting that in their recent recruitment for attack on the nitroaromatic, the original regulation has been lost and a substrate-specific regulation has not yet been acquired.

A similar assembly of diverse genes to produce a novel pathway on a plasmid has been reported for the degradation of 1,2,4-trichlorobenzene in a *Pseudomonas* strain P51⁹⁴. The initial attack on the chlorobenzene converting it to the chlorocatechol required five genes, four for the multicomponent dihydroxylating dioxygenase (*tcbAaAbAcAd*) and a single diol dehydrogenase gene (*tcbB*). These were found as a cluster (*tcbAaAbAcAdB*) in the same order and closely related to the isofunctional cluster of genes (*todC1C2BAD*) involved in the initial attack on toluene and benzene in *Pseudomonas* strain F1¹¹⁰. On either side of these genes were mutated and nonfunctional remnants of the two flanking genes from the *tod* pathway (*todF*-like gene upstream and *todE*-like gene downstream), which are not required for chlorocatechol dissimilation. This subset of the *tod*-like genes carrying remnants of their history was located between two IS elements forming a composite transposon Tn5280. This arrangement suggested recruitment into a chlorocatechol degrader of a module of *tod*-like genes from a toluene-degrader followed by subsequent mutational changes. A very similar situation has been found in a 1,2,4,5-tetrachlorobenzene-degrading *Burkholderia* strain BS12⁵, which has a very similar dioxygenase/diol dehydrogenase gene cluster, and which also carries residues of the same two inessential meta pathway genes flanking the dioxygenase genes. This unit was within a few kbp of the genes for the further catabolism of the chlorocatechol product.

Similar events leading to another strain degrading chlorobenzene *Ralstonia* JS705 were pinpointed with even great precision⁵⁴. In this strain a similar module, showing the same gene order (*mcbFAaAbAcAdBE**; *mcbE** being a functionless homologue of *todE*) as the homologous *tod* genes was located between an IS element at one end and an incomplete fragment of the same IS element at the other. This module was adjacent to genes for chlorocatechol catabolism identical in order to those found in the archetypal chlorobenzoate-utilizing strain *Pseudomonas* B13, the catabolic genes for which have recently been shown to exist on a novel mobile element involving phage-like integrases⁹⁰. A toluene-degrading strain (*Ralstonia* JS745), isolated from the same source as JS705, was shown to contain a 100% identical *mcbFAaAbAcAdBE* region found in JS705 but with a complete *mcbE* gene and without the flanking IS-like elements⁵⁴. This example appears to have identified from the same environment a very strong candidate donor strain (JS745) from which gene transfer of the dioxygenase/dehydrogenase module has taken place into JS705 to produce the hybrid pathway enabling it to use the chlorobenzene as sole carbon source.

A possible trapped intermediate state in the modular evolution of a novel pathway has been reported by Park and Kim⁶⁸ in *P. putida* HS12 able to degrade nitrobenzene but by a different pathway to that described above⁴⁸. This strain carries two plasmids pNB1 (59.1 kbp) and pNB2 (43.8 kbp) and, whereas the nitrobenzene catabolic genes *nbzA*, *nbzC*, *nbzD*, *nbzE* are located on pNB1, *nbzB* is found on pNB2. Whereas *nbzCDE* are adjacent and appear to be both operonic and tightly regulated, indicating an evolutionary mature module, neither *nbzA* nor *nbzB*, in their isolated locations, are regulated.

The ability of plasmids to transfer or be mobilised by conjugation must be an important factor in the evolutionary construction of novel pathways where the constituent genes come from different hosts, as is indicated by the results discussed above. It follows that recombination must also be an equally important element in that evolution. There is some evidence arising from sequence analysis of plasmid-encoded meta pathway sequences that homologous recombination may have taken place between, for example, two copies of meta pathway operons in the same cell, giving rise to hybrid genes or hybrid operons^{1, 6, 38}. Indeed such a recombination was demonstrated experimentally for a TOL plasmid pWW53, which carried direct repeats of two complete but distinguishable *xyl* lower pathway operons⁶⁵. Recombination between them caused a major deletion of the *xyl* upper pathway operon and a change in the catabolic phenotype of the plasmid. However it is the illegitimate recombination effected by insertion sequences and transposons that is likely to be most effective in bringing about novel reassortments of genes and therefore the major evolutionary changes in catabolic abilities. The combination of interspecific conjugal transfer by plasmids and transposable elements, which can hitch lifts on plasmids but also stop off on chromosomes or transfer to other plasmids, in theory allows sets of genes an almost unlimited access to all bacteria within a similar environment. It is therefore not surprising that in many examples of partial sequences of catabolic plasmids in the literature there are transposase-like genes in close proximity to the catabolic genes. In the majority of such reports there is no evidence as to whether or not the gene was currently functional and in some cases they were clearly not. However the presence of such genes or pseudo-genes points to the likelihood that the genes have been active at some stage in the evolutionary history of the pathway.

In addition to transposons and insertion sequences other variants of mobile DNA have been discovered some of which have been shown to carry catabolic functions and which must also participate in the processes of interspecific gene exchange. These include the *clc* chlorobenzoate-degradative element of *Pseudomonas* B13⁹¹ and the large biphenyl transposon Tn4371^{53, 83}, both of which seem to involve phage-like site-specific integration mechanisms and both of which bear close relationships with other non-catabolic mobile elements or

islands. A further example is the novel 90-kbp conjugative chromosomal element carrying biphenyl and salicylate catabolism from *P. putida* KF715⁶⁰, detailed sequence information about which is not yet fully available.

Because of the mosaic structure of catabolic plasmids and the fact that many appear to contain residues of their past history, we suggest that they probably warrant a more careful bioinformatic examination than can often be given during the annotation process when it is more important to get a quick overview than it is to get a highly detailed picture. We can present a single example of where we hit upon an unusual arrangement when we were comparing the sequence of the 'upper pathway' operon of the nitrotoluene plasmid of *P. putida* TW3 with other partial plasmid-encoded catabolic gene sequences in the database including the pWW0 sequence. Three sequences from the plasmid of 4-nitrotoluene-degrading *P. putida* TW3 (AF043544)⁴², a TOL plasmid pDK1 originating in USA (AF019635)⁴⁷ and a TOL-like plasmid from *P. putida* TMB (U41301)²⁸ originating in Italy were compared. Reanalysis of all of these, without restricting the analysis to complete ORFs (i.e., using BLASTN or BLASTX), showed there were two transposase-like genes (*tnpA1** and *tnpA2**) divergently transcribed and immediately upstream of the first genes of the operons (homologues of *xyIU*) (Figure 8A). In the TMB plasmid, upstream of that were the two regulatory genes, also divergently transcribed (homologues of *xyIR* and *xyIS*): the 5' end of the TW3 sequence also has a few codons of the 3' end of a homologue of *xyIR* suggesting that it too might have the two regulatory genes in the same position as in TMB. In strong contrast the paired *xyISR* genes on pWW0 have this same relative arrangement, but are located downstream of the meta pathway operon some 35 kbp away from their position in TMB³⁰. When we examined the pWW0 sequence between the two extreme ends of the catabolic genes and the two directly repeated copies of *IS1246* using BLASTN, we identified a homologue of *tnpA1** adjacent to *xyIR*, exactly as in TMB. The sequence of the 5' end of *tnpA2** was also present in the same location but was interrupted by *IS1246*. However the rest of the *tnpA2** was identified from BLASTN, but not as a distinguishable ORF, at the other end of the catabolic region in the same relation to *xyIU* as in TMB. This organisation, which could not be picked up by conventional annotation of ORFs, suggests that originally the *xyI* genes of pWW0 were arranged as in TMB (and by implication pDK1 and the TW3 plasmid) but have subsequently become separated by an event involving *IS1246*. A possible scenario for this is presented in Figure 8B and involves a recombination between two copies of *IS1246*, one adjacent to *xyIR* and one on a separate plasmid. Further sequence analysis using BLASTN shows an even more complex assembly since in pWW0 *tnpA1** is interrupted by two apparently complete ORFs, one of a further *tnpA* gene adjacent to an ORF of unknown function (*orf86*).

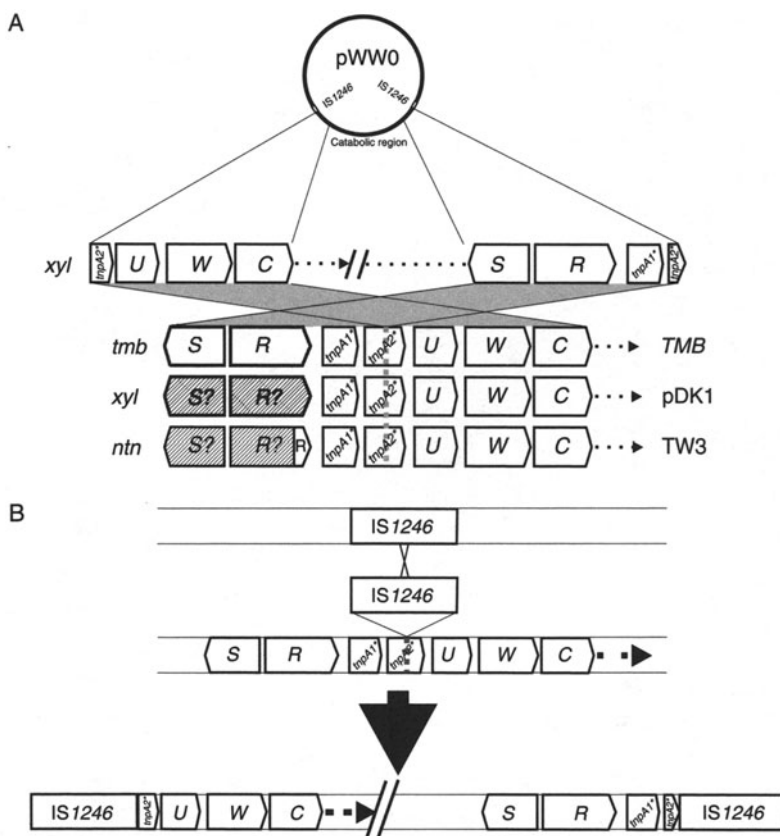


Figure 8. A. Comparison of structures of toluene and substituted toluene catabolic genes showing the difference the similar arrangement in *tmb*, *xyl* genes (on TOL plasmid pDK1) and *ntn* genes and the different arrangement of the *xyl* genes on TOL plasmid pWW0. Genes fully sequenced are shown in white and genes assumed to be present are hatched. *tnpA1** and *tnpA2** show the position of pseudogenes with homology to transposases. B. Possible mechanism by which the different arrangement on pWW0 occurred. This involves firstly the insertion of a copy of *IS1246* into *tnpA2** followed by homologous recombination between the inserted copy and another copy present on another replicon. The presence of other inserted DNA within *tnpA1** in pWW0 (not shown) indicates a more complex history of DNA exchanges.

7. CONCLUSION

The catabolism of chemicals synthesised or released into the environment by man is not only a fundamental environmental problem but, together with antibiotic resistance, presents the microbiologist with the clearest examples of how bacteria have evolved over a remarkably short period of time. As we have

tried to argue, plasmids are important elements in both these processes. To those working in the field it is disappointing that the explosion of sequencing technology has resulted in a very large number of complete bacterial genomes, both of medical and more recently of environmental significance, and yet the number of catabolic plasmids, far smaller and easier to sequence, which have been sequenced is still in single figures at the time of writing. It is to be hoped that in the time span of future *Pseudomonas* meetings this situation will be corrected and there will be many more examples that can be analysed.

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PLASMIDS OF THE GENUS *PSEUDOMONAS*

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1. INTRODUCTION

Genomic study of the genus *Pseudomonas* depends on knowledge of not only chromosomal sequences of the major species comprising the genus, but also the mobile genetic elements (MGEs) that carry key determinants within and between species. MGEs include plasmids that replicate autonomously in the cytoplasm of their host, temperate phage that can coexist with their host as prophage either integrated into the chromosome or as a plasmid, and transposable elements that can facilitate the movement of DNA between locations independently from homologous recombination. MGEs can vary in size from the smallest insertion sequence (IS) to the largest mega-plasmid (>400 kb). MGEs are often but not always associated with DNA identified as mobile or recently transferred on the basis of genomic comparisons. For *Escherichia coli*, where there are now complete genome sequences for a number of strains of this one species, it is clear that 5–10% of the genome can differ between strains. Comparisons between the genomes of *Pseudomonas* species discussed elsewhere in this volume is revealing equivalent differences. There is a scaffold of genomic functions present in all members of a species or genus but in addition there is extra DNA present in some strains but not others. In some cases the differences may be due to loss of DNA, but in many it is due to acquisition of horizontally transferred DNA. If one sums the DNA that is associated with such elements across the many strains that comprise a species this can

equal or exceed another whole chromosome. The DNA that is accessible to the whole species, while not present fully in any one strain, but that has the means to move into adjacent strains or species, is defined as the horizontal gene pool (HGP). One purpose of this chapter is to help readers think about the HGP of the genus *Pseudomonas*.

Many MGEs are associated with important phenotypic properties, such as the ability to utilise an unusual organic compound as a carbon source, to grow in the presence of one or more inhibitory substances, to invade a particular host or to cause disease. The logic governing which characteristics are MGE-associated has been the subject of much debate³⁶. However, such discussion must bear in mind that DNA found in one MGE can normally also be found in other locations and not always associated with an MGE. Therefore, the phenotypic properties and the MGE that carries them should not be seen as permanent or exclusive. Bearing this in mind the consensus appears to be that the phenotypic characteristics of MGEs should not relate to a constant parameter in the environment. Rather, it relates to a variable factor, that the bacteria may or may not encounter, as opposed to a parameter that is experienced on a regular or frequent basis. Alternatively it may be a new trait that may have existed previously in a different context and is spreading to a new microbial population or community where the phenotype may provide an advantage. A second general characteristic is that MGE-associated genes tend to exist in self-sufficient packages. That is, they can confer their phenotype in a variety of hosts without depending very critically on specific interactions with the machinery of their host except for replication and gene expression. This increases the chance of them conferring a selective advantage after transfer to the new host. In some cases this means that the genetic segment conferring the trait is quite large—encoding a catabolic pathway, for example.

As the subject of genomics develops and the variations between genomes reveal new mechanisms by which genes can be rendered “mobile,” definitions become blurred. Thus it has been suggested that pairs of essential genes that are highly conserved between many species can act like an MGE for any DNA that by chance gets inserted between them¹⁰⁰. The proposal is that the conserved genes will allow recombinational integration into the chromosome of any new host that the DNA enters so long as it carries these conserved genes, even if the general level of similarity to its genetic source is quite low. Therefore, it is important to separate MGEs into the core that provides the basis for mobility and the genetic cargo that is rendered mobile. Families of MGEs exist that consist of basically the same core with different genetic cargos.

This chapter will focus on plasmids. These are MGEs that carry a replication and maintenance system that make them independent of the host chromosome for replication and stable inheritance. They can therefore carry their genetic cargo into the cytoplasm of any species in which they are able to

replicate autonomously and thus avoid the constraints of homologous recombination. Each type of MGE will have a certain host-range where these generalisations apply. However, it is commonly the case that the host-ranges of a transfer system and of a replication/maintenance system are not identical. The MGE transfer system may provide the ability to spread into a host where establishment of the property depends on some sort of integration into the chromosome—that is, the MGE naturally acts as a suicide vector.

Although MGEs may simply provide random genetic combinations that are then subject to selection, the logic of what evolves will depend on the properties of the MGEs that are present initially to fuel the process of variation. For example, it is clear that not all IS sequences move randomly and therefore the outcomes of IS movement can be biased. Knowing the bias in a system can therefore allow one to make sense of genomes that are uncovered and make it possible to predict some aspects of how the system may develop in future. In order to provide a way of thinking about this, one needs to catalogue and annotate this HGP in a way that is useful and accessible.

The cataloguing of *Pseudomonas* plasmids has not proceeded uniformly. This is partly due to the complexity of functional incompatibility tests, designed to determine whether two plasmids share replication and stable inheritance functions, and should be placed in the same group (see Sections 2 and 8). The basis of such a test is to ask whether two distinguishable plasmids coexist stably as if they were on their own, or whether they increase the rate of segregational loss of each other when introduced into the same cell line. Performing such a test becomes difficult when plasmids and host already carry many selectable markers and the number of incompatibility groups grows. In addition, the replacement of functional incompatibility tests by hybridisation to probe sequences to place plasmids in families has reduced the need to determine incompatibility type, although for environmental studies, knowing which plasmids compete directly with each other is of major importance. The ideal would be to know both sequence family and incompatibility grouping. It is pleasing to note that this is being recognised, and in many cases incompatibility tests are being performed now to check whether plasmids related by sequence are compatible or incompatible. This chapter will provide an update on previous reviews of this sort and aims to bridge the gap between sequences and incompatibility groups, providing some generalisations from the data that we have now accumulated.

2. PLASMID GROUPS IN *PSEUDOMONAS*

Table 7.1 lists the plasmid groups known in *Pseudomonas* species as well as plasmids whose replication system is sequenced and which may represent the archetypes of new plasmid groups. It can be seen that this includes

Table 1. Representative plasmids of *Pseudomonas* incompatibility groups.

Incompatibility or homology group and group attributes	Plasmid	Size (kb) ^a	Phenotype, comments	References
IncP-1 (IncP) Integrated regulation system Broad host range Conjugative Group divided into alpha, beta and gamma subgroups	RK2 (RP1, RP4)	60	Cb Km Tc IncP1α subgroup	[92] ^b
	R751	53	Tp IncP1β subgroup	[129] ^b
	pQKH54	73	Hg IncP1γ subgroup	[58]
	pADP-1	96	atrazine catabolism IncP1β subgroup	[1] [87] ^b
	pB4	79	Ax Em Sm IncP1β subgroup	[122] ^b
	pB10	65	Ap Sm Su Tc Hg IncP1β subgroup	[102] ^b
IncP-2 Conjugative, narrow host range Typically very large All confer resistance to tellurite	pMG1	450	Bo Gm Hg Sm Su Tc Uv Bacteriophage inhibition, pyocin inactivation	[63] [65]
	pBS271	450	0ε-caprolactam degradation. Tc	[13]
IncP-3 (IncA/C) Broad host range Conjugative	pBS73 RA1	88 130	Cm Km Sm Su Tc Hg Su Tc	[12] [5]
IncP-4 (IncQ) Broad host range Mobilisable Strand displacement replication Small size High copy number	IncQ1α IncQ1β	8.7 5.1	Sm Su cryptic	[104] ^b [140] ^b
	IncQ1γ IncQ2α	11 12	Cm Km Sm Su Carries glutathione reductase and MerR homologues	[112] ^b [96] [25]

Multiple essentially compatible subgroups ^c	IncQ2 β	pTC-F14	14	Unknown	[43]
IncP-5 Large, conjugative, narrow host range	Rms163		220	Bo Cm Su Tc	[99] [120]
IncP-6 (IncG) Broad host range	Rms149		57	Cb Gm Sm Su Mobilisable	[55]
IncP-7 Narrow host range	Rms148 pCAR1 ^d		180 199	Sm Carbazole/dioxin metabolism	[99] [83] ^b
IncP-8	FP2		90	Hg Pm	[94]
IncP-9	pWW0 (TOL)		115	Toluene catabolism Conjugative	[49] ^b
Unstable in <i>P. aeruginosa</i> and non- <i>Pseudomonas</i> spp. at 43°C	pM3		75	Sm Tc	[51]
	NAH		80	Conjugative Naphthalene catabolism	[32] [139]
	pDTG1		83	Naphthalene Catabolism Conjugative	AF491307
IncP-10 Narrow host range	R91		48	Cb Conjugative	[66] [89]
IncP-11	RP1-1 (R18-1)			Cb	[61]
	R151		33	No detectable extrachromosomal DNA Cb Gm Km Sm Su Tb	[16] [93]
IncP-12 Narrow host range	R716		165	Sm Hg	[15]

Table 1. Continued

Incompatibility or homology group and group attributes	Plasmid	Size (kb) ^a	Phenotype, comments	References
IncP-13				
Narrow host range	pMG25	99	Bo Cb Cm Gm Km Sm Su Tb	[64]
	pQM1	252	Hg Uv Conjugative	[6] [40]
IncP-14				
Broad host range	pBS222	17	Tc Conjugative	[12]
pPT23A-like ^e	pPT23A	100	Coronatine synthesis	[47]
Narrow host range	pFKN	40	Conjugative	[10]
Compatible and largely homologous plasmids often found in one strain			Virulence (against plants)	[98] ^b
pIP02-like	pIP02	40	Cryptic, but carries multiple orfs of unknown function.	[123] ^b
Conjugative, broad host range				
Unclassified <i>P. fluorescens</i> sugar beet group I	pQBR11	294	Hg Conjugative	[79]
Unclassified <i>P. fluorescens</i> sugar beet group II	pQBR24	307	Hg Conjugative	[79]
Unclassified <i>P. fluorescens</i> sugar beet group III	pQBR55	149	Hg Conjugative	[79]
Unclassified <i>P. fluorescens</i> sugar beet group IV	pQBR30	263	Hg conjugative	[79]
Unclassified <i>P. fluorescens</i> sugar beet group V	pQBR61	64	Hg conjugative	[79]
Unclassified (not P-1 or P-4)	pAM10.6	10.6	Phenol catabolism	[95]
Narrow host range				
Unclassified	pKB740	8.1	2-Amino-benzoate metabolism	[3]
Rolling circle replication(?)				
Unclassified	pPP8-1	2.53	X66604 Cryptic	[60] ^b

Rolling circle replication				
Unclassified	pPS10/ pECB2 pRA2	10.0/ 4.48 33	Cryptic	[90] [21] [77] ^b
Unclassified			Restriction-modification system mobilisable	
Unclassified	pRO1600	3	Cryptic	[91] [138] [62]
Unclassified	pVS1	30	Hg Su Mobilisable, narrow host range	[56] ^b

Resistances: Ap, Ampicillin; Ax, Amoxycillin; Bo, Borate resistance; Cb, Carbenicillin; Cm, Chloramphenicol; Em, Erythromycin; Gm, Gentamycin; Hg, Mercury; Km, Kanamycin; Pm, Phenyl-mercuric acetate; Sm, Streptomycin; Sp, Spectromycin, Su, Sulphonamides, Tb, Tobramycin; Tc, Tetracycline, Te Tellurite; Tp, Trimethoprim; Uv, Ultraviolet light.

^aWhere no size estimate in kilobases could be found, size was calculated as $1.5 \times$ (molecular weight in MDa).

^bPaper reporting or finishing the complete plasmid sequence.

^cSubgroups as given by Rawlings and Tietze⁹⁷.

^dTyped by sequence homology.

^eThis grouping defined by homology; contains mutually compatible plasmids.

plasmids across the whole size range—from pPP8-1 at 2.5 kb⁶⁰ to the IncP-2 mega-plasmids that have been estimated to be as large as 400 kb or more⁶⁵. Although some plasmids are cryptic, that is, they carry no known phenotypic determinant, others carry one or more traits such as antibiotic resistance, heavy metal resistance, or degradative functions. There do not appear to be reported cases of plasmids carrying both antibiotic resistance and degradative determinants, but mercury resistance is certainly found on both sorts of plasmids, and it would be surprising if there are no examples that carry both types of function, given the past widespread use of antibiotics in an agricultural context.

The primary basis of the grouping is the incompatibility groups defined in the 1970s⁹⁹ and subsequently expanded slightly by work in the laboratories of George Jacoby and Alexander Boronin. Related plasmid replicons typically cannot be co-maintained, and are said to be incompatible, forming an "Incompatibility Group." At present there are 14 listed plasmid incompatibility groups in *Pseudomonas*^{12, 64}, but our list (Table 1) includes additional groups or individual plasmids that have not yet been given an IncP number. We propose not to add any more group numbers until the relation between unclassified plasmids and the defined groups is known, a task that is currently underway. The basis for the main classification is still the functional incompatibility test for displacement of one plasmid by another where they carry different selectable or screenable markers. This is obviously a labour-intensive activity compared to hybridisation or PCR screening with specific primers but such genetic tools are not yet available for the majority of *Pseudomonas* plasmids. If the pool of plasmids were the same across all bacterial genera then the tools available in one group of organisms should be applicable to others. However, although such tools have been developed for *E. coli* plasmids by isolation of mini-replicons and DNA sequencing²⁶, they are of only limited applicability to *Pseudomonas*. Thus, except in the case of plasmids with a known broad host range, the hybridisation tools from the Couturier collection have largely failed to be useful for classifying plasmids from soil, marine or river samples^{18, 28, 31, 57, 71, 114, 117, 131, 135}. This suggests that the plasmids of the *Pseudomonas* groups either belong to phylogenetic families completely different from those found in *E. coli* or are generally too distantly related to be detectable by cross-hybridisation. A key point is that clinical isolates formed the major source of the plasmids for the Couturier collection. Recent sequencing of plasmids from nonclinical environments show that most plasmids carry a replicon recognisable by its similarity to previously characterised systems, but often only with a low degree of sequence identity. Therefore the second of the two possibilities seems more likely to be correct.

Thus there is a need for more complete nucleotide sequences for plasmids found in *Pseudomonas* to underpin their screening and classification. Currently there are complete sequences of archetypes of only three *Pseudomonas*

plasmid Inc groups: IncP-1 (RP4, R751, pADP-1, pB4, pB10); IncP-4 (RSF1010, pDN1, pIE1130); and IncP-9 (pWW0, pDTG1). Very recently it has been possible to allocate a fully sequenced plasmid, pCAR1, to IncP-7 on the basis of incompatibility testing (Hideaki Nojiri, The University of Tokyo, personal communication) as well as by similarity to the partial sequence of a known IncP-7 plasmid (ASH and CMT, unpublished). This thus allows us to place pL6.5 (part of whose sequence is in the database, AJ250853, but for which there is no published account) from *P. fluorescens* in the IncP-7 or IncP-7-like group. There are also complete sequences of a number of additional plasmids that either originate in *Pseudomonas* or have a sufficiently broad host range that they can exist either stably or semi-stably in *Pseudomonas* sp. but have not been assigned to a particular group. The most notable of these are the plasmids pIP02, pSB102 and pXF51, which we recently identified as a new plasmid group, at least some of which appear to have a broad host range^{86, 103, 123}. A separate broad host-range plasmid that has been completely sequenced is RA3⁷⁶. It may be that these plasmids do belong to known groups, but at present they cannot be assigned to one. The absence of DNA probes or DNA primers for PCR amplification for all the known groups makes it difficult to perform the classification easily. We have started a programme to create the tools to underpin the creation of such a catalogue. This process will be helped by the existence of sequences for additional replicons, namely those of pAM10.6 (95), pPP8-1⁶⁰, pPS10/pECB2^{21, 90}, RA1, which belongs to the IncP-3 broad host-range group that is equivalent to the IncA/C group in *E. coli*⁸⁰, pRO1600⁶⁷ and pVS1⁵⁶.

In some cases the plasmids fall into groupings that have been studied in some detail, but where the relationship with any of the originally defined Inc groups is not known. One example of this is the pT23A family, which includes at least 14 plasmids¹⁰⁵. Although the level of sequence identity between all plasmids in the group is quite high, it is known that naturally occurring strains often carry more than one member of the family, suggesting that between certain members there is sufficient divergence that incompatibility is effectively zero¹⁰⁶. These plasmids play an important role in the range of adaptive and virulence properties possessed by *P. syringae*¹³⁷. A second example is the five groups of pQBR plasmids from sugar beet rhizosphere that confer mercury resistance in *P. fluorescens*⁷⁹. This set of groups is not related by sequence apart from carrying mercury resistance and are expected to carry distinct cores of replication and transfer systems. Sequencing studies are underway with these plasmids and two segments that promote replication of a replicon-probe vector have been isolated, but there is not yet enough information to place these in one of the known families or into new families. We have included these on the list with the long-term aim to establish whether such a group is completely separate or belongs within one of the families defined by the

incompatibility groups that are already listed. It is therefore important to have the sequences of at least mini-replicons from representative plasmids for the various IncP groups. These will be used to check the diversity within the known incompatibility groups and to place unclassified plasmids into one or other of the known groups or a new group on this basis.

This leads on to the final issue about classification, which is illustrated by the IncP-4 plasmids, but also by the pT23A plasmids referred to above. In the IncP-4 group it is known that there are at least three different incompatibility types within the same sequence family. Rawlings and Tietze⁹⁷ suggest that the subdivisions in the family be referred to as IncP-4 α and IncP-4 β by analogy with the IncP-1 plasmids, although in that group all the plasmids are incompatible with each other. Although we have stuck to the published nomenclature, our suggestion is that for a family of plasmids related by replicon sequence, but containing a number of incompatibility groups, the major subdivisions based on sequence phylogeny should be referred to as A, B, C, etc. Within these branches, incompatibility groups could be referred to as .1, .2, .3, etc. and then further subdivisions within an incompatibility group be referred to as α , β , γ , etc.

3. WHAT DO WE MEAN BY PLASMIDS OF *PSEUDOMONAS*?

Our aim is to define those plasmids (as a subgroup of Mobile Genetic Elements) that play an important role in the HGP of *Pseudomonas* species. However, it is worth considering whether the fact that an MGE can be found in *Pseudomonas* automatically should mean that it should be classed as a *Pseudomonas* MGE. The genomes of different organisms have different characteristic G + C content, codon usage and other sequence motifs, that appear to impose themselves with time on any acquired DNA. This is the basis for identifying horizontally acquired DNA and estimating how long it is since the DNA arrived in the species. Genomes are mosaics of different DNA segments. There is generally a core of essential functions interspersed with segments acquired from different sources at different stages. Plasmids are no exception to this, and one important way of characterising newly sequenced plasmids is to determine whether they appear to be uniform in genomic motifs or obviously put together from widely different origins.

One of the first analyses of a plasmid genome in this respect was carried out with the genomic sequence of IncP-1 α plasmid RP4/RK2^{92, 141}. The driving force behind the study was to test the idea that the IncP-1 plasmid backbone has been selected to be deficient in restriction endonuclease cleavage sites as the result of encountering restriction barriers during spread from

one strain to another. What became clear was that the backbone was deficient in some palindromic hexamer sequences and very rich in others. To some extent this could be due to high G+C content in the plasmid genome that is 61.75%. However, randomly generated sequences with a similar G+C content did not give the same frequency of sites as in RP4, suggesting that selective pressure has been responsible for the distribution of sites in RP4. Those hexamers that were underrepresented corresponded to recognition sequences for restriction endonucleases that have been catalogued in the *P. aeruginosa* cluster of *Pseudomonas* species. Thus bias against the presence of such sites as a result of the selective pressure created by restriction barriers is a plausible explanation. In addition, the plasmid genome showed the same high frequency of GCCG and CGGC motifs that are characteristic of *P. aeruginosa*. It is therefore possible to deduce that RP4 evolved within *P. aeruginosa* or an organism very like it. In contrast, while the IncP-1 β plasmids have a similar, if not higher, G+C content than the IncP-1 α plasmids, they do not have the same preponderance of GCCG/CGGC motifs, suggesting that the evolutionary context in which they evolved was different¹²⁹.

It is therefore useful to know whether a plasmid found in *Pseudomonas* has been part of the genome for a long time or is a new arrival. To count as an ancient *Pseudomonas* plasmid one can therefore argue that its replication and maintenance system should have characteristics of *Pseudomonas* genomes. Its genetic cargo may have been acquired by forays into other genera, but unless the core has *Pseudomonas* characteristics then it should probably be regarded as a transient, even though this may simply mean that it has not been a resident long enough to acquire the *Pseudomonas* genomic motifs. As discussed in Section 8 below, relatively small changes can be responsible for allowing a plasmid to be able to replicate and be maintained efficiently in a new host, even if it was not stable to start with. It would therefore be possible for a plasmid to be reasonably well adapted to a new host, but not have the genomic characteristics of that host. Under these circumstances it should be regarded as a recent *Pseudomonas* plasmid. If we apply this analysis to the plasmid groups listed in Table 1, we can categorise the various plasmid groups. The plasmids that we would class as genuine *Pseudomonas* plasmids on this basis are: IncP-1 (61–64%), IncP-4 (61%), IncP-9 (59%); pIP02-like (61%), pPS10/pECB2 (60%), RA2 (60%), pVS1 (60%). Interestingly the IncP-3, IncP-7 and the pT23A replicons have G+C contents of 53–54%, much lower than would be expected if they are typical of the *Pseudomonas* genome. Plasmids pAM10.6 and pRO1600 have a slightly higher, but still not typical G+C content (56%). The IncW plasmids have in the past been classed as broad host range and included in the list of *Pseudomonas* plasmids, but in addition to their G+C content being atypical (57%), they are not stably maintained in *Pseudomonas* in the absence of selection and therefore have not been

included in our list. However, it is interesting to note the similarities with the *rep* genes of some of the *Pseudomonas* plasmids listed (see Section 6 below) and the similarity of organisation of the transfer regions of IncP-9 plasmids to those of the IncWs. In addition, the G+C contents of IncP-9 (57.4%) and IncW (56.9% at the region level) transfer regions are very similar. Clearly the relationships between plasmid replicons and their host genomes are of great interest and the accumulating sequences should make this a productive area for bioinformatic studies, which is beyond the scope of this chapter.

4. ISOLATION OF PLASMIDS

Although a very large number of plasmids have been isolated and at least partially characterised it is not yet clear whether the plasmids that have been characterised are fully representative of those plasmids that carry important phenotypic determinants in natural environments. There is therefore a growing effort to sample new environmental niches and determine the nature of plasmids that are present. Obviously the most direct approach (referred to as endogenous isolation) is to spread environmental samples on appropriate types of solid media and purify to single colonies bacteria that grow. Plasmid screening can then be performed either without purification of plasmid DNA, for example, by the Eckhardt technique³⁷ in which bacteria are lysed in the gel well and plasmid DNA is induced to migrate away from the very high molecular weight chromosomal DNA, or after plasmid extraction by one of the standard methods that may be adapted to isolation of high molecular weight plasmids^{54, 69}. However, this relies on being able to grow a fully representative range of the bacteria that potentially harbor plasmids. It also is limited to finding bacteria that make up at least 0.1% or more of the population because screening thousands of cultures for plasmids may not be practical¹¹⁷. Attempts to use endogenous isolation to recover putative IncP-9 plasmids from pig manure samples, which had tested strongly positive for these plasmids on the basis of PCR screening of total DNA extracted, proved negative⁷⁴.

Since it is known that many bacteria observed by microscopy in environmental samples are unculturable, approaches that do not rely on culturing the bacterial host have been devised—the so-called exogenous isolation. The first such approach, called bi-parental exogenous isolation adds a new selectable and culturable recipient bacterial strain to the environmental samples, incubates the mixture for long enough to allow plasmid transfer to take place, and then selects derivatives of the added recipient that have acquired a new marker from the environmental sample^{6, 7}. This procedure can in principle capture both conjugative plasmids and mobilisable plasmids that reside in a

host that also carries a self-transmissible plasmid that can mobilise the plasmid carrying the selected trait. These studies are particularly relevant to this review because the hosts that have been used successfully for such isolations include *P. aeruginosa*, *P. putida* and *P. fluorescens*. Examples of acquired traits are resistance to mercury⁷⁹, antibiotic resistance¹¹² and ability to degrade herbicide¹³³. This has been a useful approach but is limited to plasmids that carry the selectable marker that has been chosen.

An alternative approach is to use what is termed tri-parental exogenous isolation^{58, 131, 135}. In this approach two general plasmid properties are selected—the ability to transfer and the ability to mobilise a second plasmid. To the environmental sample are added a plasmid-free selectable recipient bacterial strain (strain A) as well as a second strain (strain B) carrying a mobilisable plasmid with a selectable marker. After enough time for plasmid transfer to take place the mixture is plated on medium to select for transfer of the mobilisable plasmid to strain A. This should only occur if a self-transmissible MGE has transferred to strain B and allowed the mobilisable plasmid to transfer to strain A. In many cases the new plasmid also transfers to strain A resulting in recipients carrying both plasmids. Recipients of the selected marker are then screened for acquisition of new environmental plasmids. Again *P. putida* has often been used as the recipient in such experiments.

5. USE OF PCR PRIMERS AND ASSOCIATED PROBES FOR *PSEUDOMONAS* PLASMIDS

As mentioned above, one important aim is to develop sets of PCR primers that can be used to test whether a sample contains DNA belonging to a specific plasmid group. This could be applied to purified plasmid DNA, to total DNA from a pure culture, or total DNA from an environmental sample that may contain not just a mixture of bacteria, but also DNA from other sources. This allows one to analyse a sample without the need to cultivate the bacteria carrying the plasmid. The power of this technique was originally demonstrated with IncP-1 (IncP), IncP-4 (IncQ), IncN and IncW plasmids⁴⁸. Reconstruction experiments indicate that the detection limit of this method is somewhere in the region 1 bacterium carrying a particular plasmid in 10^3 to 10^4 bacteria. Before such primers can be used it is important that they are shown to give a product reproducibly with complex samples containing DNA of a particular plasmid group and that a product is not obtained with DNA from plasmids of other groups. Although the presence of a PCR product of the right size can indicate the presence of the query sequence, in practice it is always necessary to use Southern blotting or DNA sequencing to check that the PCR product does correspond to a template of interest. It is surprising how

often a PCR product of the right size is obtained that turns out to be spurious when it is cloned and sequenced. However, if a product is obtained that does not hybridise to the probe then it may also be that the DNA that gave the product comes from an MGE that is only distantly related to the archetype—that is, it diverges too much to give cross-hybridisation. Such a situation would be interesting because it indicates the identification of novel relatives of known plasmids that may help to fill a gap in the continuity of a plasmid group.

Such primers are currently available for the groups IncP-1^{48, 128}, IncP-3/IncA/C⁸⁰, IncP-4⁴⁸, IncP-9^{50, 74}, IncN and IncW⁴⁸ and pIP02-like¹²³. In the near future we should be able to add probes for IncP-6 and IncP-7. The previously described primers have been used to screen the available environmental samples and this will allow a comprehensive assessment of their distribution and abundance^{48, 74, 113}. Interestingly, while the plasmids that we have designated as typical of *Pseudomonas* were found in a range of water and soil samples, the IncN and IncW plasmid signals were confined largely to manure samples suggesting a closer link to enteric flora, reinforcing the categorisation already suggested above.

6. PLASMID REPLICATION

Since plasmid typing is based on the relatedness of the replication and maintenance systems, these aspects of the plasmids will be the major focus of the rest of this chapter, with plasmid transfer being covered much less thoroughly. Plasmid copy number and size tend to be inversely related in all bacteria and *Pseudomonas* species are no exception. Most attention has focused on the large, low copy number plasmids, but small high copy number plasmids are known and some have been studied, particularly with a view to vector development. The aim of this section is not to give a detailed description of replication, but to illustrate the diversity and relationships between the systems in *Pseudomonas* plasmids. The reader is referred to previous reviews for more information on replication *per se*³⁸.

The smallest DNA segment that has been reported to promote replication in *Pseudomonas* is only 640 bp in size and originated in *Acidithiobacillus ferrooxidans*, but can replicate in quite a number of Gram-negative genera including *Pseudomonas*⁷⁰. Interesting its G+C content is 59%, consistent with the criteria defined above as typical of the *Pseudomonas* genome. How it replicates is not known since it bears no resemblance to other replication systems and encodes no protein. It has only been studied as part of a shuttle vector and so it is conceivable that it is not capable of self-replication, that is, it may only function in conjunction with the *E. coli* plasmid to which it is joined.

Relevant to this point is the 300 bp replicon derived from the *P. fluorescens* megaplasmid pQBR11 that also encodes no protein¹³⁶, and seems very unlikely to be the *bona fide* replicon for such a large plasmid. The hint from other sequences in the databases that the pQBR segment might promote rolling circle replication would not make sense in terms of the size of the plasmid, which is estimated to be about 300 kb. We suspect that when pQBR11 is analysed in more detail or completely sequenced it will reveal a more conventional replicon, and that at least some replicon-capture strategies may potentially give artefacts. An additional replicon was isolated in the same way, on a 4.9 kb *Hind*III fragment from pQBR55¹³⁴. It has some of the characteristics of a replicon such as the presence of a *dnaB*-family (helicase) gene, a GC skew inversion (which indicates the transition from one replication direction to another) and a gene related to *traA* of IncP-1 α that make sense if it is the mega-plasmid replicon. However, this conclusion needs to be confirmed before we can be sure that it really is a genuine plasmid replicon.

A 2.5-kb rolling circle replication (RCR) plasmid was isolated from *P. putida* P8⁶⁰. The predicted Rep protein and the putative replication origin placed the replication system firmly in the pC194 family of RCR plasmids. Not only does the plasmid lack additional maintenance genes that would be needed if the plasmid became very large and forced the copy number down, but also the accumulation of single stranded DNA for such an expanded plasmid would result in recombinational instability that would prevent growth in plasmid size. A second plasmid, pKB740 from *Pseudomonas* sp.³, may also replicate via a RCR strategy⁶⁰, although this conclusion depends on similarity to the putative *rep* gene of a relatively poorly characterised bacteriophage¹¹⁸. It is interesting to note that the Rep protein of pWW0 and pM3 has weak but apparently significant similarity to a sequence group that includes RCR Rep proteins^{49, 51}. We can speculate that the IncP-9 Rep originally drove RCR but evolved in such a way as to lose nicking activity and acquire the ability to recruit host replication functions.

The Rep of pT23A plasmids is related to that of ColE2, a small, medium copy number, DNA PolI-dependent plasmid of *E. coli*⁵⁹. The reason for the PolI dependence is probably not that the Rep protein leads in DnaA, but rather processes a transcript going through the replication origin, creating an R-loop that allows PolI to initiate replication, but would not allow PolIII to do so. Since pT23A plasmids are large and relatively low copy number plasmids, it is not clear to what extent the details of the replication system will match those of ColE2, despite the similarity of the Rep proteins.

All other groups of *Pseudomonas* plasmids for which sequence is available contain replicons that depend on at least one Rep protein to activate a replication origin containing multiple Rep binding sites, termed iterons. We can group these replicons by the relationships between their Rep proteins. It is

possible to put almost all of these proteins, including the ColE2 Rep into a single alignment and build a phylogeny^{29, 38}. While some subdivisions as outlined below are very clear, the relationships between the less closely related are much more difficult to be sure of and so are not discussed here. The Rep proteins from IncP-1 plasmids (product of the *trfA* gene), the IncP-4 plasmids (product of the *repC* gene) and RA2 (product of the *repA* gene) each fall into separate groups found only on closely related plasmids. These plasmids all carry other stability functions belonging to clearly defined and broadly distributed families. The diversity of the Rep functions may thus reflect the drive of plasmids to avoid competition with each other by having a unique, or at least not cross-reacting Rep-*oriV* system.

IncP-3 Rep is similar to Rep proteins of plasmids from *Buchnera aphidicola* and *A. ferrooxidans*. IncP-7 Rep is related to that of many other plasmids⁸³ including *Pseudomonas* plasmids pPS10/pECB2 (which are very similar to each other) and pRO1600. This group also includes the Rep proteins from the FIA replicon of IncFI plasmids, RepE, for which there is a crystal structure⁷², as well as the Pir protein of R6K and Rep from pSC101 that have also been studied in detail^{42, 75, 82, 107}. A general point that should be noted from this group is that a family of Rep proteins can be found in narrow host-range plasmids from a range of different species that is much wider than the host ranges of the individual systems. That is, the various members of the Rep family are adapted to different narrow groups of hosts. IncP-9 Rep shows similarity to the Rep proteins of pBBR1 from *Bordetella pertussis*^{4, 51} and pL10.6 from *P. fluorescens*⁹⁵. This set seems to be an odd one out because as mentioned above sequence alignments place it in a group of Rep proteins from RCR plasmids, although admittedly the similarity is low. The Rep proteins from the pIP02-like group are most closely related to the IncW Rep from plasmid pSa¹²³. Finally pVS1 shows similarity to Reps from plasmids of *Paracoccus alcaliphilus*⁸ and *Erwinia stewartii*⁴¹.

The general model for the actions of these Rep proteins is that they bind to the replication origin and to assist host proteins to activate it³⁸. The normal sequence of events is DnaA binding along with the plasmid Rep, followed by localised unwinding. This can allow the DnaBCD helicase to enter and unwind the origin further, which enables primase to initiate leading strand synthesis. Replication origins normally contain an A+T-rich region that is proposed to be where the double-stranded DNA melts first. In most *oriVs* it is also possible to identify potential DnaA boxes, and where it has been investigated directly DnaA has generally been confirmed at these sites. In the case of IncP-9, DnaA binding cannot be detected when present on its own under standard conditions, but does bind when Rep is present (R. Krasowiak and CMT, unpublished).

The most complicated replicon is that of the multicopy IncP-4 system that is the only one to date known to require three plasmid-encoded proteins—an iteron-binding protein (RepC), a helicase (RepA) and a primase (RepB)¹⁰¹. The replication strategy employed is called strand displacement because there are no lagging strand origins to assemble the primosome that normally generates Okazaki fragments. Instead the replication origin has two divergent leading strand origins that can fire simultaneously or sequentially. The replisome that assembles continuously displaces the nonreplicated strand. A practical consequence of this is that the system seems to be inherently unsuited to replicating a large plasmid, because as the plasmid gets larger, the increased delay in converting the 'lagging' strand to dsDNA by the action of the other leading strand origin, results in accumulation of ssDNA. This can be recombinogenic and so large derivatives of IncP-4 plasmids are structurally unstable, a fact that came to light most forcibly when cosmid cloning vectors based on the IncP-4 replicon, were found to lose inserted DNA (often up to 40 kb) during genomic library construction. There is the possibility that the IncP-7 replicon includes a helicase since there is a gene downstream of *rep*, and possibly cotranscribed, that shows sequence similarity to a helicase from *Salmonella enterica*⁸³, but as yet no experimental evidence either way is available.

As can be seen therefore, apart from a few exceptions referred to at the start of this section, we can make predictions about the functions of most of the replication systems in *Pseudomonas* plasmids described to date. We expect that as other large plasmids are discovered and characterised, they will be found to have replicons related to those described above.

7. PLASMID MAINTENANCE

The organisations of the replication and stable inheritance regions of *Pseudomonas* plasmids that are known to encode additional stability determinants are summarised in Figure 1. The clustering of replication and maintenance systems is a way of maximising co-inheritance during plasmid recombination and transfer¹²⁴. The most extreme example of this is the IncP-1 plasmids where the majority of the plasmid is a core of coordinately regulated replication, maintenance and transfer functions^{92, 129}. Many other plasmids demonstrate this principle as well. For example, comparison of the pWW0 sequence⁴⁹ with that of the naphthalene degradation plasmid pDTG1¹¹⁹, shows that the conserved IncP-9 core is the clustered replication, maintenance and transfer region, outside which the plasmids diverge more extensively. The purpose of this section is to summarise the maintenance systems found in *Pseudomonas* plasmids. The three major categories of these factors are

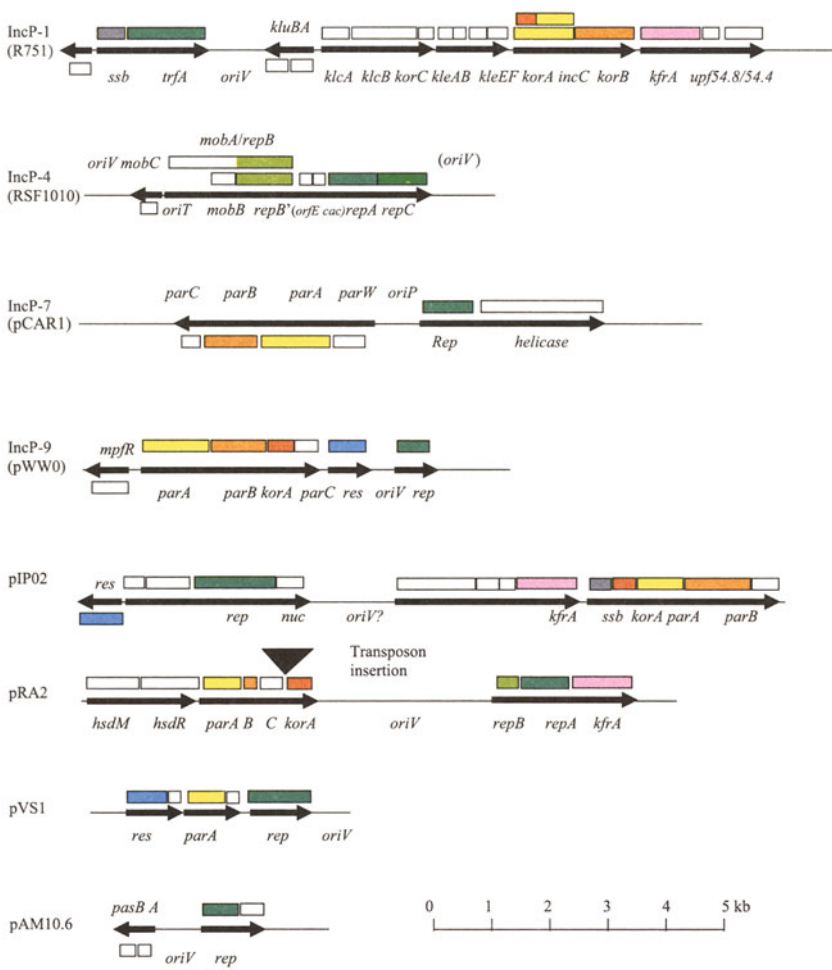


Figure 1. Summary of the core organisation of the replication and stable inheritance regions from major plasmid groups discussed in the chapter. The summary excludes those plasmids for which there is only a replication origin and a *rep* gene sequence (IncP-3, pRO1600, pPS10/pECB2). Orfs running from left to right are shown above the line and orfs running right to left are shown below. Heavy arrows on the line indicate transcriptional units. In general orfs coding for proteins of the same family are shown in the same colour. Orfs coding for *rep* genes are shown in green, even though they may not all belong to the same family. Where there are more than one *rep* gene in a single plasmid, they are shown in different shades of green. For IncP-1 and IncP-9 the start of the mating pair formation apparatus genes is shown at the left end of the cluster. The IncP-1 *trfA-oriV* region is shown as continuous rather than interrupted as it is in most naturally occurring plasmids because it is possible to deduce the ancestral organisation by aligning sequences from the IncP-1β plasmids R751 and R906¹¹⁵.

multimer resolution, active partitioning and post-segregational killing. For an overview of how these systems work readers should consult more specialised reviews⁴⁴.

Apart from RCR plasmids (that should generate monomeric products even from a dimeric template as a result of their mode of replication) plasmids are generally thought to need a multimer resolution system. This generates monomers from the dimers that arise by recombination¹²¹. That multimer resolution is vital for plasmid stable inheritance has been most extensively demonstrated for ColE1 through study of its *cer* system⁵². Resolution is generally achieved by site-specific recombinases, that prevent the accumulation of dimers and higher-order multimers. The most complicated multimer resolution system (*mrs*) is encoded by the IncP-1 α plasmids where three genes (*parCBA*) out of a set of five (*parABCDE*) transcribed from two divergent operons encode multimer resolution³⁴. Interestingly this block of genes is located physically away from the rest of the replication and stable inheritance functions and appears to have been acquired as a block after creation of the IncP-1 regulon, since it is not present in the IncP-1 β plasmids¹²⁹. It is the ParA of this system that shows sequence identity to the well-characterised resolvase protein family and most other plasmids just seem to encode an homologue of ParA if they have an identifiable putative *mrs* function. That ParA alone can provide multimer-resolution activity is consistent with the observation that the RK2 *parA* plus its associated cis-acting site can give plasmid stabilisation, at least in some strains³⁴, although greater stabilisation was found when *parB* and *parC* were included. The plasmid pIP02 also encodes a ParB homologue, close to but not contiguous with *parA*. However, the importance of either of these functions for pIP02 is unknown since *parA* was inactivated by the cassette that was inserted to label the cryptic plasmid¹²³. Other plasmids with *res* homologues includes IncP-9, but does not include IncP-1 β plasmids. Surprisingly, deletion of *res* from the IncP-9 mini-replicon in pMT2 did not result in increased loss rate or apparent accumulation of multimers⁵¹, even after UV irradiation which might be expected to elevate recombination rates. The plasmid pVS1 also encodes a resolvase that does not appear to be necessary for stable inheritance⁵⁶. The inconsistency in whether or not a resolvase gene is found leaves us wondering whether the need for such a function is actually such a universal property of plasmids.

The second type of general stability determinant is the active partitioning system. It is generally only the low copy number plasmids that carry active partition systems, and the presence of such a system allows one to functionally define plasmids as low or high copy number, as well as by the yield of DNA obtained during screening. The IncP-4 plasmids appear to fall into the high copy number category. This was clearly demonstrated by inserting extra copies of the *oriV* iterons into the plasmid causing copy number to

be reduced and resulting in decreased segregational stability as expected if segregation is random⁹.

The active partitioning systems that have been recognised on *Pseudomonas* plasmids all fall into the type I *par* family, all of which have a Walker-type ATPase as their ParA^{11, 45}. These systems can be subdivided, not just by sequence similarity, but also by organisation of the *par* locus, the presence of a putative DNA-binding domain at the N-terminus of the ParA protein, the family to which the ParB protein belongs and the location of the *parS* sequence, which acts as the centromere-like sequence for the partitioning process. The classic P1 and F systems are designated Ia, while those in which ParA does not have a DNA-binding domain, does not have a classic ParB protein and has its *parS* upstream rather than downstream of the *par* operon are referred to as Ib. The *par* systems of the IncP-1, IncP-7, IncP-9 and pIP02-like systems currently fall outside either of these two major groups both organisationally and phylogenetically. The IncP-1, IncP-9 and pIP02 systems are more closely related at the protein level to each other than they are to other systems, but the IncP-7 *par* system is quite distinct. The pRA2 system belongs to type Ib, as may the system based on the *parA* gene of pT23A. The plasmid pVS1 is peculiar because knock-out of its putative *parB* gene apparently has no destabilising effect, as cited in the database annotation. The ParA and ParB homologues in the IncP-1 system appear to be closer to their chromosomal counterparts, particularly from *P. aeruginosa* and *P. putida*, than to other plasmid-encoded Par proteins. Additional genes have been designated *par* on the basis of coregulation with *parA*, *parB* genes but further evidence for a direct role in partitioning is lacking. This includes *parC* of pWW0 (*tolA* in pM3), *parW* and *parC* in pL6.5 and pCAR1.

Although not common, post-segregational killing systems are found and in other cases may so far have been overlooked. The best characterised are the *parDE* systems of IncP-1 α plasmids⁶⁸ and the *pas* system found in the IncP-4 plasmids⁹⁷. A *pas* system is observed on pAM10.6⁹⁵ while a restriction-modification system which should be able to act as a *pks* system is found on RA2⁷⁶. Thus the repertoire of maintenance functions observed in the *Pseudomonas* plasmids is exactly what we would expect from our general knowledge of plasmids that have been studied in detail. However, there are still many associated genes whose role if any has not yet been defined. Of these, the role of the KfrA-like proteins, first identified in the IncP-1 plasmids, and that bear resemblance to the Smc proteins (structural maintenance of chromosomes) is most intriguing². Hopefully the accumulation of information on other *Pseudomonas* plasmids will add to our ability to identify additional gene families that are conserved across many groups and that this will justify further work to establish the role that they play.

8. DETERMINANTS OF INCOMPATIBILITY

Knowledge of what determines incompatibility is useful because it can help to focus attention on key areas during the analysis of new plasmid genomes. Incompatibility normally can be observed to arise between two plasmids in the same bacterium, where the plasmids are phenotypically distinguishable but which are indistinguishable with respect to at least one of the key mechanisms that results in their stable inheritance: replication, post-segregational killing or active partitioning. If two plasmids share effectively identical replication and replication control systems, then random selection from a pool of plasmid molecules to be replicated will result in a bias toward one or the other type, which will be reinforced by subsequent selection until cell lines with only one type breed true. If they share a post-segregation killing system then it can only protect against loss of both plasmids from the cell—that is, loss of just one of the plasmids is ignored because the other plasmid can still provide the antidote that prevents the toxin from killing the bacterial host. Thus the shared *pks* negates the effectiveness against plasmid loss, for both plasmids. If the two plasmids share an active partition system based on a pairing step that is not locked into the replication cycle, then random selection from the pool of plasmids will again result in the loss of effectiveness of the partitioning system—daughter bacteria will always get one plasmid, but not necessarily both plasmids.

Does this background fit with what we find in practice in *Pseudomonas* plasmids? For the IncP-1 plasmids the replication origin and the iterons that it carries are the primary incompatibility determinant^{125, 127} with the partitioning and control functions of the *korA-incC-korB* operon playing a secondary role^{88, 126}. The contribution of the *parDE* region (which encodes a post-segregational killing system) to incompatibility has been assessed by placing the *parD* gene in trans and showing that this can destabilise RK2. However, the strength of the effect is strain-specific, reflecting the relative importance of the different stability determinants in different host backgrounds^{35, 110}. The *klc-kle* region has been shown to be important for stability in *P. aeruginosa*¹⁴³. While studying this effect it was discovered that a series of direct repeats when present in both of a pair of plasmids caused incompatibility in *P. aeruginosa* but not in *E. coli*¹⁴².

For IncP-4 plasmids incompatibility appears to be due to the iterons that bind RepC in the replication origin. Some IncP-4-like plasmids carry an extra, nonfunctional copy of the *oriV* region, located downstream of *repC* sequences⁹⁷. While the extra *oriV* is not activated by the RepC protein of the plasmid it is in, it does bind RepC from other IncP-4-like plasmids, thus interfering with the replication of the second plasmid. This appears to be a strategy to displace other IncP-4-like plasmids. This does cause a problem for the

IncP-4 classification system, because it means that plasmids whose Rep proteins clearly fall into different specificity groups are placed in the same group because of the one-way effect of the nonfunctional *oriV* region. Where we understand the basis for such effects, we propose that the unidirectional incompatibility should not be used as the basis for primary classification, but should be regarded as a secondary effect.

For IncP-9 plasmids the replication origin region alone is not able to express incompatibility, but needs to be combined with the *rep* gene⁵¹. A second region corresponding to the putative centromere-like region associated with the *parAB* region of the mini-replicon plasmid pMT2 from pM3 also expresses incompatibility very strongly, at least in *E. coli*⁵¹. This may correlate with the importance of this region in stable inheritance of the plasmid and the fact that the *oriV*-*rep* region is not able to function as an independent replicon. These observations show clearly that incompatibility is not due only to replication determinants but can also be associated with stable maintenance functions and thus can be usefully used as an indication of regions that are important for stable inheritance.

9. DETERMINANTS OF HOST RANGE

A number of plasmid groups known in *Pseudomonas* have a broad host range and in a number of cases are already included also in the plasmid classification system of *E. coli* (IncP-1, IncP-3, IncP-4, IncP-6). There is some discussion currently about what constitutes a broad host range and it has been proposed that this should mean at least that the plasmid is able to establish itself and then be maintained reasonably stably without selection in at least two of the subdivisions of proteobacteria. Thus a plasmid that can shuttle between *P. putida* and *E. coli* may not necessarily fit this definition since both these are within the gamma subdivision. On this basis pWW0 does not count as broad host range whereas the well-characterised IncP-1 (IncP in *E. coli*) and IncP-4 (IncQ in *E. coli*) do, along with the less well-characterised IncP-3 (IncA/C in *E. coli*) and IncP-6 (IncG in *E. coli*). Given the diversity of species within the *Pseudomonas* genus and the importance of transporting genes from other genera into *Pseudomonas*, the factors that determine whether a plasmid can establish itself in a *Pseudomonas* species and be maintained there stably is an important property. Conversely, the factors that limit the maintenance of *Pseudomonas* plasmids outside the genus are also of interest.

That it is the replication and stable inheritance system that is particularly important in determining host range was shown by hybrid plasmids created between IncP-1 plasmid RK2 and *E. coli* IncFI plasmid F⁵³. By transferring the IncP-1 and IncFI replicons from *E. coli* to *P. aeruginosa* with either the

IncP-1 or the IncFI transfer systems it was shown that although the F transfer system is not as efficient as the IncP-1 transfer system, the absolute limitation on host range is the replication system. The IncFI transfer system could mobilise the IncP-1 replicon to *P. aeruginosa*, but when the IncP-1 transfer was used to move the IncFI replicon efficiently from *E. coli* to *P. aeruginosa* it could not replicate at all. A similar conclusion was reached with respect to the IncI α plasmid, that is, the plasmid could be transferred to species in which it could not replicate¹⁴. There is therefore considerable interest in determining what are the factors that limit the host range of a plasmid's replication and maintenance system.

For the IncP-4 plasmids it seems clear that the major factor in its broad host range is the multiple Rep proteins that it encodes, giving it a degree of independence from the machinery of its host. It not only provides the machinery to prime leading strand synthesis, but also dispenses with the need for lagging strand synthesis, by its strand displacement replication strategy¹⁰¹. It is more of a challenge to explain those plasmids that on the face of it are very similar to each other, possessing both a *rep* and *oriV* but which have different host ranges. The IncP-1 plasmids have been studied in this respect as a broad host-range plasmid while pPS10 has been studied as a narrow host-range plasmid. For these plasmids it appears that it is the macromolecular interactions between the plasmid and host replication machinery that determine the host ranges.

The minimal replicon of IncP-1 plasmids consists of the *oriV* region and the *trfA* gene encoding proteins that in conjunction with host DNA replication proteins activate *oriV*. Clues about the host range came from the discovery that the *rep* gene *trfA* encoded two related Rep proteins, TrfA-1 and TrfA-2, from alternative translational starts in the same open reading frame^{109, 116}. Subsequently it was shown that TrfA-2 was sufficient to drive plasmid replication in *E. coli* and *P. putida* but not in *P. aeruginosa*^{33, 108}. Conversely the sequences needed to provide a functional *oriV* in different species were not identical. In *P. aeruginosa* a segment encoding only the minimal five TrfA-binding iterons, plus the adjacent A+T and G+C regions is sufficient for (albeit slightly less efficient) replication, whereas in *E. coli* and *P. putida* the region containing DnaA-binding sites next to the TrfA iterons is necessary²⁷. Subsequent biochemical analysis has shown that both TrfA and DnaA are necessary to activate the replication origin and that TrfA helps DnaA to recruit additional host factors necessary to activate the replication origin^{19, 20, 30, 73}. The implication is that recruitment of host factors in *P. aeruginosa* can occur without a specific DnaA-binding site, and that this extra activity of TrfA depends on the protein domain(s) present in TrfA-1 but not in TrfA-2. Thus the TrfA complex is flexible enough to cope with species differences, but this does depend on there being two forms of the TrfA protein—it may be that TrfA-1 does not work as well as TrfA-2 in some species.

The results with RK2 highlight the importance of interactions between the plasmid and host encoded parts of the replication machinery. That the differences in the interactions necessary in different species can be quite small is suggested by studies on how a *Pseudomonas* plasmid of limited host range can extend its host range. Plasmid pPS10 from *P. syringae* can replicate in *E. coli* at 30°C but not at higher temperatures³⁹. This may be because many *Pseudomonas* species exist mainly in environments where the average temperature is in the region of 10°C to 25°C and so the systems are optimised for lower temperatures. For example, temperature adaptation has been documented for plasmid transfer in aquatic environments⁷. However, it may also reflect less robust interactions with host replication factors to which the plasmid is not adapted. Such plasmid temperature-sensitivity is not unique—it is also observed for the IncP-9 plasmid pM3¹³⁰. To understand the limitations on interactions with the host it was possible to isolate mutations that were able to suppress this temperature-sensitivity. Such mutations could occur in either the plasmid or the host. Those in the plasmid mapped in the *rep* gene^{39, 84} while those in the host mapped to the *dnaA* gene⁸⁵. This demonstrates that the host range of a plasmid is not fixed and can be altered by small changes in key proteins.

In addition to replication, the stability of a plasmid in different species can be an important determinant of its effectiveness as a carrier of genetic information in different host populations. The way that a host can modulate effectiveness of stable inheritance functions is illustrated again by the IncP-1 plasmids. For example, the effectiveness of the *parABC* gene varies from strain to strain in both *E. coli* and *P. aeruginosa*³⁵ and so one should expect that it may also vary from species to species as well. The stability conferred by the *kle* region of RK2 is also host specific—apparently being much more important in *P. aeruginosa* than in *E. coli*¹⁴³. Another factor that may create host-specific variations in the effectiveness of replication and stable inheritance genes is the strength of gene expression signals. For example, not all bacteria recognise promoter sequences in the same way. However, for the IncP-1 plasmids it is suggested that this is directly counteracted by the complex auto-genous control circuits, which use very strong promoters repressed by autoregulation to compensate for contexts where the “standard” signals work less effectively¹²⁴.

10. PLASMID TRANSFER

One of the key features of MGEs is their ability to spread from one bacterium to another either as a result of genes encoded by the plasmid itself, or provided by helper genes on a different replicon present in the same cell.

Transfer can occur by transformation, transduction or conjugation, and the context determines which of these is dominant. With the large plasmids of Gram-negative bacteria it is generally expected that transfer will be by conjugation, but in fact transformation is more widespread a process than often realised^{81, 111}. This is illustrated by pRA2, which appears to transfer by a transformation process since it is blocked by DNase I⁷⁷. It appears that DNA is released from donors and is then taken up by recipients in the immediate vicinity. Surprisingly, pRA2 DNA seemed to be much more efficiently acquired by the recipients than DNA of control plasmids, suggesting that pRA2 may promote its uptake through DNA structure or an expressed function.

The most studied mechanism for active transfer is conjugation, which relies on two sets of functions: For Mating Pair Formation (Mpf) and DNA Transfer and Replication (Dtr)¹⁴⁴. Many small plasmids carry only the Dtr functions, which are then termed *mob*, for mobilizable, since they require a conjugative system to mobilise them. A coupling protein is needed to allow a Dtr system to harness a Mpf system and the specificity of such coupling proteins determines which plasmids are efficiently mobilised by which self-transmissible plasmids¹⁷. The best characterised interaction among the *Pseudomonas* plasmids is the efficient mobilisation of the IncP-4 plasmids by IncP-1 plasmids. The most widespread gene set responsible for pilus biosynthesis and mating pair formation apparatus belong to the *virB/trb* family that is a subgroup of the type IV secretion systems (T4SS)²⁴. Such genes are found on the IncP-1, IncP-9 and the pIP02-like plasmids. The equivalent apparatus of pCAR1, the putative IncP-7 plasmid, shows much more similarity to the transfer system of plasmids from *Enterobacteriaceae*, most notably Rts1 (IncT)⁸³. The DNA processing systems associated with these Mpf systems are not always the same. Thus the IncP-9 Dtr system is like the IncW system but is quite distinct from the IncP-1 system. The Dtr systems include all those functions that are needed to transfer once a mating bridge has been formed—assembly of the relaxosome, association with the mating bridge, triggering of nicking and initiation of transfer rolling circle replication, and then reconstitution of the plasmid in the recipient cell by lagging strand synthesis and maturation. The transfer origin where nicking occurs, *oriT*, is the core of the Dtr system and there is recognisable sequence similarity between all *oriT* sequences¹⁴⁴. All the self-transmissible or mobilisable *Pseudomonas* plasmids described to date have a recognisable *oriT* sequence.

One topical area of research is the visualisation of biological processes proceeding in real time. For the IncP-1 β plasmid this has been achieved by tagging the plasmid with multiple copies of the *lac* operator. The plasmid is then allowed to transfer into a recipient that is expressing a fusion protein consisting of the *lac* repressor protein LacI and the green fluorescent protein from the jelly fish *Aequoria victoria* that is commonly used as a reporter⁷⁸. The

fluorescent protein is distributed evenly in the recipient, until the plasmid enters and then binds to the plasmid and forms a clear focus that eventually replicates and starts to segregate symmetrically. An alternative way of visualising transfer more generally (without being able to see the plasmid foci inside the bacteria) involves tagging the plasmid with a *lacp-gfp* cassette so that GFP production can be switched off in donor bacteria by the presence of *lacI^q* in the chromosome²³. Expression is then switched on in the recipient after transfer due to the absence of *lacI* gene, allowing easy detection of the cells that have acquired the plasmid. This has been applied to pWW0 because it is the best studied degradative plasmid and has been used as a model for manipulation of bioremediation strategies that involve environmental release of plasmid-bearing strains. Therefore pWW0 transfer in microbial biofilm communities has been studied²³. The results showed that plasmid transfer does occur when a donor interacts with a growing or established biofilm, but that transfer to endogenous bacteria only occurs at the interface between the donor and recipient cultures and does not spread throughout the recipient population²². The importance of plasmid transfer systems in promoting biofilm formation by their hosts has been discovered recently⁴⁶ and this raises a number of important issues about key aspects of conjugation. The most important of these stems from the observation that F plasmid can stimulate biofilm formation even in the absence of recipient F⁻ bacteria. It appears that interaction between plasmid-positive bacteria is not inhibited under conditions of biofilm formation despite plasmid-encoded surface exclusion functions. Thus plasmid transfer into plasmid-positive bacteria may occur at reasonable frequency, allowing homologous recombination between related plasmids to occur. Therefore in natural environments conjugation may promote interplasmid recombination and thus favour rapid plasmid evolution. Evidence that this is indeed occurring may come from current projects to sequence multiple members of the same plasmid family. If recombination turns out to be a frequent event then it will be an important factor of the HGP.

11. ASSESSMENT OF THE PHENOTYPIC CARGO OF NEW PLASMIDS

One important dimension of the genomic studies described here is the ability to assess the genetic cargo carried by the plasmid stable inheritance and replication systems. Where the core of the plasmid is essentially identical it would be sensible to have a strategy that avoids re-analysis of this core. Any approach depends on knowledge of what is core and what is cargo. A powerful way of doing this has been described recently and applied to IncP-1 plasmids. After isolation of new IncP-1 plasmids, plasmid DNA was purified, subject to

restriction, the fragments separated by electrophoresis, Southern blotted and then probed with DNA corresponding to the known IncP-1 core. Restriction fragments that did not light up were then extracted from similar gels and then cloned and sequenced³¹. In this particular study a number of functions not previously associated with IncP-1 were identified, including restriction/modification, superoxide dismutase and multidrug efflux system of the RND family.

12. CONCLUSIONS

The production of this chapter comes at a time of considerable activity in this area of research. Sequencing of many *Pseudomonas* plasmids is underway, including archetypes of the major incompatibility groups. Unfortunately much of this data is not yet available. However, when it is we will not only have a clearer picture of the diversity of the HGP in this genus, but the overall scheme of plasmid groups may look considerably simpler. Also, the methods are now available to screen DNA from crude samples for sequences characteristic of different plasmid types and to capture plasmids from such samples. The increase in sequence information will therefore provide the tools to extend this screening to the full range of *Pseudomonas* plasmid groups.

The aim of cataloguing the plasmids of *Pseudomonas* is to provide a means of investigating the HGP of *Pseudomonas* and ordering the information that we have about it. This may first help us to see patterns in the information and eventually provide an explanation for them. Some correlations are already obvious. For example, in surveying the plasmids that carry degradative functions, it seems that IncP-2, IncP-7 and IncP-9 are dominant among those carrying well-established catabolic pathways for naturally occurring organic molecules, whereas IncP-1 β plasmids are prevalent among those with newly acquired ability to degrade man-made compounds¹³². The tools that allow us to screen for plasmids of known groups and capture plasmids of new groups will hopefully ensure that the information that we possess is representative of the real world. This should establish whether generalisations like the above are due to our limited information or reflect real properties.

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PHAGES OF *PSEUDOMONAS*

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1. INTRODUCTION

A large number of bacteriophages have been isolated and analyzed in *Pseudomonas*. Many of them have been used as tools for epidemiological studies (phage typing) and for genetic analysis by transduction⁴⁰. Some have been developed as molecular biological tools for *Pseudomonas*; for example, the D3112 transposable phage-derived cloning system¹⁵, ϕ CTX-derived integration-proficient vectors³⁷, and the D3 phage-derived cosmid vector⁹⁵. Furthermore, infections of some temperate phages have been demonstrated to confer new phenotypes to the host strains (lysogenic or phage conversion), and thus played important roles in generating the genetic and phenotypic diversity of *Pseudomonas*^{30, 38, 64}. From the clinical point of view, virulent phages have been regarded as potential therapeutic tools for *Pseudomonas* infections, the treatment of which are often very difficult because of their high resistance to antibiotics^{103, 104}. Despite such importance of bacteriophages in various aspects, only limited knowledge about the biological and genetic features of *Pseudomonas* phages were available, though several RNA phages and filamentous phages have been extensively studied as model systems, for replication, gene regulation, morphogenesis, and structural biology of the macromolecules. However, by the recent progress in sequencing technology, it is now easy to obtain the whole genome sequence information of bacteriophages, which has considerably expanded our knowledge on the biological features of *Pseudomonas* phages.

Although the genome sequences of several *Pseudomonas* phages with small genome sizes, such as a filamentous phage Pf3, were determined very early⁶⁷, the progress in the genomic analyses of *Pseudomonas* phages was relatively slow compared to enterobacterial phages. In 1999, however, the genome sequence of ϕ CTX was determined for the first time as a tailed double-stranded DNA phage of *Pseudomonas*⁸⁰. After that, three more tailed double-stranded DNA phages each belonging to different phage family have been determined. Furthermore, the presence of several prophage or phage-related elements that are integrated into the chromosomes of *P. aeruginosa* strain PAO1 and *P. putida* strain KT2440 were identified by whole genome sequencing^{82, 102}.

In this chapter, we first briefly summarize the genomic features of *Pseudomonas* phages with small single-stranded RNA, double-stranded RNA, and single-stranded DNA genomes. Then, genomic features of recently sequenced tailed double-stranded DNA phages will be described in more details. Finally, genomic features of prophages on the two sequenced *Pseudomonas* genomes, including those of the genes for phage-tail like bacteriocins (R-type and F-type pyocins), are summarized. Many transposable phages have also been isolated and analyzed in *Pseudomonas*. In particular, bacteriophage D3112, which has a 38-kb linear double-stranded DNA genome and possesses a genetic organization similar to a transposable coliphage Mu, has been extensively examined^{43, 54}. However, we do not include D3112 in this chapter, since only a part of its genome sequence has been determined so far^{4, 108}.

2. SINGLE-STRANDED RNA PHAGES

Bacteriophages of the *Leviviridae* family are small phages that possess positive, single-stranded RNA genome¹⁰⁹. They infect a wide range of Gram-negative bacteria that express F or polar pili on the cell surface. Initially, these bacteriophages were isolated from *Escherichia coli*, but were found soon after in *Pseudomonas* as well^{8, 24, 116}. Since then, many single-stranded RNA phages have been isolated from various bacteria. Single-stranded RNA phages have a common genomic organization and a high degree of similarity in the replication and translational control mechanisms. Based on the different physical and serological properties, they are categorized into four groups (I–IV), each represented by coliphages MS2, GA, Q β , and SP. Because of the simple structure, these phages have been studied as model systems for replication and translation. In particular, their coat proteins serve as excellent model systems for studying protein–RNA interaction and the role of secondary structure in gene regulation.

Among the *Pseudomonas* single-stranded RNA phages isolated so far, only PP7, which was originally isolated in 1966 by Bradley⁸, has been sequenced⁸⁷. PP7 is not male-specific, but instead adsorbs on the sides of polar

pili of *P. aeruginosa*⁹. The genome of PP7 (3,588-nucleotide long) is similar in size and gene organization to that of the group I coliphages. Four identified open reading frames (ORFs) were assigned as maturation, coat, replication, and lysis proteins, based on the homology to the group I phages. The structure and function of the PP7 coat protein have also been extensively studied^{62, 63, 106}

3. LIPID-CONTAINING, DOUBLE-STRANDED RNA PHAGES

The members of family *Cystoviridae* are enveloped double-stranded RNA viruses that infect bacteria⁷⁷. *Pseudomonas* phage $\phi 6$, which was discovered by Vidaver *et al.* in 1973¹¹⁰, is the best characterized member of this family, and its natural host is *Pseudomonas phaseolicola*¹¹⁰. The structure of $\phi 6$ is very unique and different from those of other lipid-containing phages belonging to the *Corticoviridae* and the *Tectiviridae* families; it contains a genome composed of three double-stranded RNA segments packaged in a procapsid covered by a viral protein shell and a lipid membrane containing several viral proteins⁹⁴. The genome of $\phi 6$ has been sequenced^{26, 72, 75}, and the lifecycle and structure of the phage have been extensively examined^{11, 16, 76, 92}. It infects the host cell by attaching to the type IV pilus that is retracted so that the viral membrane can fuse with the outer membrane of the host. The fusion results in the nucleocapsid being located in the periplasmic space. Digestion of the peptidoglycan by viral muramidase (protein P5) leads the nucleocapsid particle to a closer association with the host inner membrane, and the nucleocapsid successively enters the cell⁷⁷. The procapsid composed of proteins P1, P2, P4, and P7 has the ability of packaging RNA, synthesizing minus strands to make double-stranded RNA, and transcribing the genome. Plus-strand transcripts are each packaged into a procapsid in a precise and controlled process, which results in three genomic segments being in each phage particle. The filled procapsid then acquires a shell of protein P8, and further a lipid-containing membrane in the cell.

Three genome segments of $\phi 6$, which are called segments L, M, and S according to their sizes, are 6,734, 4,063, and 2,948 bp in length, and contain 5, 4, and 4 genes, respectively. The total genome size of $\phi 6$ is thus 13,739 bp⁷⁵. The packaging signals are localized to about 200 nucleotides in the noncoding regions near the 5' ends of the plus strands²⁷. Recently, the genome sequence of $\phi 8$, another member of the family *Cystoviridae*, has been determined⁴¹. The sizes of the three segments (7,051, 4,742, and 3,192 bp for segments L, M, and S, respectively) are similar to those of $\phi 6$, and the genomic organization also to that of $\phi 6$. However, there is no similarity in either nucleotide sequences or the amino acid sequences, with the exception of

the motifs characteristic to phage RNA polymerases. Furthermore, $\phi 8$ attaches directly to the rough LPS⁷⁸, and consequently, the host range of $\phi 8$ extends to rough strains of *Salmonella*. This indicates that $\phi 8$ is a member of the family *Cystoviridae* distantly related to $\phi 6$.

4. FILAMENTOUS PHAGES WITH SINGLE-STRANDED DNA GENOMES

Bacteriophages belonging to the *Inoviridae* family are filamentous phages that contain a circular single-stranded DNA genome. They can infect only bacteria bearing retractile pili, such as F-pilus. They do not kill their hosts, but instead, continue to produce virus for a considerable period of time⁷⁹. After infection, the single-stranded phage DNA (plus-strand) is converted to a double-stranded form (RF) by the action of host factors. The initial double-stranded RF molecule serves as the template for transcription to synthesize phage proteins, and then a single-stranded virus DNA and a relaxed double-stranded RF DNA are produced under the control of both phage- and host-encoded functions.

Although many filamentous phages have been isolated, they are divided into two classes based on the structures of their protein coats⁷⁰. The best-studied are F-specific coliphage f1, and its close relatives, fd and M13. They are very similar to one another, differing only at a few nucleotide positions, and are collectively referred as Ff. Another well-studied, class I filamentous phage whose genome has been sequenced is coliphage IKE, which is specific to the IncN plasmid. Its genome organization is very similar to that of Ff, but the DNA sequence homology is about 55%⁹¹.

Among the class II filamentous phages, two *Pseudomonas* phages, Pfl and Pf3^{74, 105}, have been studied well, and their genome sequences have been determined^{36, 67}. Pf3 is specific to the *P. aeruginosa* strains harboring IncP-1 plasmid, while Pfl is specific to a pilus of *P. aeruginosa* strain PAK¹⁰¹. The first sequenced Pf3 genome was 5,833 bp in length, on which nine ORFs were identified⁶⁷. The genome organization resembles that of Class I phages, but virtually no homology was found between their DNA sequences or amino acid sequences. The genome of Pfl is 7,349 bp in length, much larger than Pf3 and slightly larger than Ff (6,408 bp)³⁶. The G+C content of Pfl (66.5%) is closer to the host *P. aeruginosa* chromosome (66.6%) than that of Pf3 (45.4%). Fourteen ORFs were identified on the genome, 13 of which were confirmed to have been expressed. The genomic organization of Pfl again exhibits some resemblances to that of other filamentous phages, but the genome sequence differs from that of either Pf3 or the class I phages.

5. LIPID-CONTAINING PHAGES WITH DOUBLE-STRANDED DNA GENOMES

Bacteriophage PM2 is the only described member of the *Corticoviridae* family^{1, 21}, and is the first bacteriophage that was shown to contain lipids in the virion. It was isolated from sea water together with its host *Pseudoalteromonas espejiana* BAL-31, which was originally called *Pseudomonas* sp. strain BAL31²². It has an icosahedrally organized protein capsid surrounding a spherical inner membranous core composed of phospholipids and proteins, and a circular double-stranded DNA genome resides inside this core^{23, 50}. This structural feature resembles that of *Tectiviridae* family members, such as coliphage PRD1⁵, but the genome of the *Tectiviridae* family is a linear double-stranded DNA⁶.

The genome of PM2 is 10,079 bp in size⁶⁹ and contains 21 putative genes. Among these, genes *I–IX* encode the structural proteins P1–P9. Protein P1 forms the receptor-binding spike structure at the vertices of the virion, and P2 is the coat protein. Proteins P3–P9 are associated with the membranous core. Gene *XII* probably encodes a replication initiation protein. These PM2 genes are organized into three operons; the leftward transcribing early operon (OEL), the rightward early operon (OER), and the late operon (OL)⁶⁸. The OEL operon encodes two transcriptional repressors, P15 and P16; P15 controls its own promoter and P16 the OER operon. The OL operon is positively regulated by the concerted action of P13 and P14, which are coded in the OER operon. Interestingly, P14 has a zinc finger motif similar to the transcription factors from *Eucarya* and *Archaea*⁶⁸.

6. TAILED DOUBLE-STRANDED DNA PHAGES

6.1. P2-like *Myoviridae*: Cytotoxin-Converting Phage ϕ CTX

ϕ CTX is a temperate phage isolated from a *P. aeruginosa* strain, which produces a toxin called cytotoxin³⁰. Cytotoxin is a protein toxin originally identified by Scharmann⁹³. It is produced as a dimeric, inactive form (procytotoxin), but turns into a monomeric, active form by proteolytic removal of a short C-terminal peptide^{31, 84}. On a eukaryotic target cell, the active toxin forms a transmembrane pore by probably oligomerizing into a homopentamer⁸⁵. The toxin thus belongs to the pore-forming toxin family. The cytotoxin gene (*ctx*) is present on the ϕ CTX genome, and *P. aeruginosa* strains are converted to cytotoxin-producers by lysogenization with ϕ CTX³⁰.

As described before, ϕ CTX was the first completely sequenced tailed double-stranded DNA phage from *Pseudomonas*⁸⁰. The ϕ CTX genome is a linear 35,538-bp double-stranded DNA with 21-bp 5'-extruding cohesive ends, and encodes 47 ORFs. Among these, 15 gene products have been identified in the phage particle by the microsequencing analysis of the virion proteins⁸⁰. The *ctx* gene is located at the very left end of the genome, just 361-bp downstream of the left cohesive end³². The *attP* site and the integrase gene (*int*) of ϕ CTX is located within the 2-kb region on the opposite side of the genome, and by using this integration system, ϕ CTX is integrated into the 3'-end of the serine tRNA gene on the host chromosome³². This tRNA gene is located at the position 2947583–2947672 of the *P. aeruginosa* PAO1 chromosome. PAO1, however, is not sensitive to ϕ CTX because ϕ CTX recognizes only a limited range of LPS core oligosaccharide structures as the receptor¹¹⁵.

There is an extensive genome similarity between ϕ CTX and coliphage P2 and its related phages (Figure 1A). Many ϕ CTX ORFs (25 out of 47 ORFs) exhibit high homology to P2 gene products with 30–65% amino acid sequence identity. The genetic organization of the ϕ CTX genome is also very similar to that of the P2 phage family^{7, 12}. In particular, the genes for morphogenesis are nearly completely conserved, clearly indicating that ϕ CTX is a member of the P2 phage family. Only the genes for replication and lysogeny, and a part of the lysis gene cassette are replaced. Several common features shared by ϕ CTX and P2-like phages, such as morphology (both belong to the family *Myoviridae*), genome sizes, use of the LPS core as receptor, Ca^{2+} -dependent receptor binding, use of the tRNA genes as the *attB* sites, and non-inducibility, further support their close relatedness^{7, 32, 80, 115}. On the other hand, the average G+C content of the ϕ CTX genome is 62.6%, which is slightly lower than that of the host *P. aeruginosa* chromosome and very different from that of P2 (50%). However, the base composition of the ϕ CTX genome is not constant, and several markedly AT-rich regions are present (Figure 1A). The remaining regions exhibit almost the same G+C content as that of *P. aeruginosa* chromosome (about 66%), and genes on these genome regions show a very good codon adaptation to *P. aeruginosa*. Taken together, it was concluded that ϕ CTX is a P2-like phage well adapted to *P. aeruginosa*, and it provided very clear evidence for the spread and evolution of bacteriophages among different bacterial genera (intergeneric spread and evolution).

As mentioned above, the ϕ CTX genome contains several AT-rich regions that are apparently of foreign origin. They include the region containing the *ctx* gene (Figure 1A). P2 also contains several AT-rich regions, they encode nonessential genes of foreign origin; *old*, *tin*, *fun(z)*, and Orf30, the first three of which are known to confer phage resistance to the lysogenic hosts¹². These foreign DNA elements on phage genomes, which are expressed as transcription units independent from the phage propagation, are now called

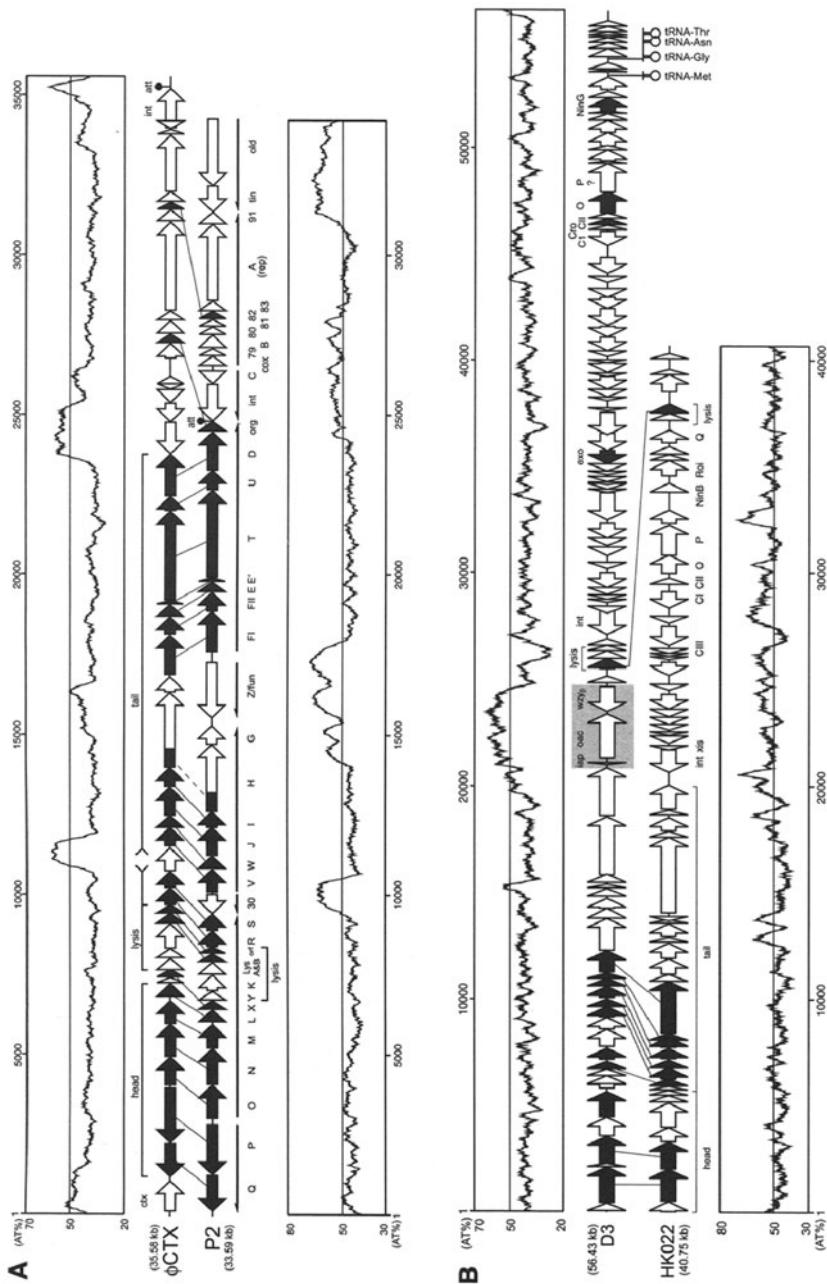


Figure 1. Genomic comparison of ϕ CTX and P2, and D3 and HK022. A. The genomes of ϕ CTX and coliphage P2 are compared. Genes exhibiting sequences homologous are connected by lines, and indicated by dark shading. B. The genomes of D3 and a lambda-like coliphage HK022 are compared. Genes exhibiting sequences homologous are connected by lines, and indicated by dark shading. D3 genes that exhibit sequence homologies to those on other lambda phage family are also indicated by dark shading. The D3 genomic region containing serotype converting genes is highlighted. Since the *iap* and *wzyB* genes were not identified in the original sequence (accession no. AF165214), the gene organization of the region is depicted from the data reported by Newton *et al.*⁸³.

“morons”³⁴. By genomic comparisons of P2-like phages including ϕ CTX, several hot spots were identified for such foreign DNAs on the P2 phage family genome. A good example is the region encoding Orf30 on the P2 genome (Figure 1A). At exactly the same position, ϕ CTX contains a DNA segment encoding a gene of unknown function but apparently of foreign origin. The region containing the *ctx* element is also one of such regions because a retron element is located at the same position on the genome of another P2-like coliphage, ϕ R67¹⁷. From the same point of view, the region encoding genes for tail fibers and their assembly (*H* and *G* genes, respectively) is also of considerable interest. The N-terminal portion of gpH, which is thought to bind to the tail body in P2²⁸, is highly homologous between ϕ CTX and P2, while the C-terminal portion of gpH, which constitutes the distal part of tail fibers involved in the binding to the host cell receptor, is not. The entire gpG sequences also display no similarity. This structural difference is in good agreement with the different host range of the two phages. More interestingly, the region encoding the C-terminal portion of gpH and gpG is one of the AT-rich regions in both phage genomes, implying that both phages have evolved by exchanging the regions with some other phages. This may be one of the key genetic events for the intergeneric spread of P2-like phages. Tail fiber proteins of double-stranded DNA phages are often made of several modules with a variety of combinations²⁸. The gpH also exhibits a modular structure both in ϕ CTX and P2^{28, 80}.

Another intriguing feature of ϕ CTX is that ϕ CTX is a member of the R pyocin-related phages that are genetically related to R-type pyocins³³. Another cytotoxin-converting phage, PS21, which contains a genome very similar to that of ϕ CTX but with some minor differences, also belongs to the R pyocin-related phage family³³ (T. Hayashi, unpublished data). It infers that cytotoxin-converting phages constitute a subgroup of the R pyocin-related phage family of *P. aeruginosa*.

6.2. Lambda-like *Siphoviridae*: Serotype-Converting Phage D3

Bacteriophage D3 was originally isolated from a clinical *P. aeruginosa* strain by Holloway *et al.* in 1960³⁹. They subsequently noted that lysogenization of the phage caused a change in the serological properties of the host cell³⁸. Later, Kuzio and Kropinski⁶⁰ demonstrated that the O-antigenic polysaccharide side chains are chemically altered in the lysogen, PAO1(D3); the hydroxyl group at the D-fucoseamine residues became acetylated, and the bonding between trisaccharide repeats changed from α 1–4 to β 1–4. These structural alterations resulted in the change in serotype from O5 to O16/20.

Simultaneously, the lysogen became resistant to superinfection by D3. Although this phenomenon is very similar to those observed for *Salmonella* phage ϵ 15^{10, 65, 66}, D3 genes responsible for the seroconversion were identified only after the whole genome sequence was determined⁵².

Bacteriophage D3 is a member of the B1 (isomeric head) morphogroup of family *Siphoviridae*. It possesses a head (55 nm in diameter) and a long flexible tail. The genome is a linear double-stranded DNA of 56,426 bp with 9-bp 3'-extended cohesive ends, and encodes 90 ORFs and four tRNA genes. The average G+C content is 57.8%, significantly lower than the host genome. In particular, the region containing three serotype conversion genes is considerably AT-rich (Figure 1B), suggesting that the region has arisen by horizontal gene transfer from another bacterium or phage with lower G+C content (see below). Since the virion morphology, virion size, and genome size of D3 was very similar to coliphage lambda and its relatives, D3 has been thought to be a member of the lambdoid group. This assumption was verified by the genome sequence; at least 16 gene products exhibit sequence homologies to those from lambda and/or lambda-like phages, and the genomic organization is similar to that of lambda-like phages with an exception of the location of the lysis genes. The lysis gene cassette of D3 is located between the tail fiber and lysogeny genes, while in other lambda-like phages, it is located downstream of the anti-terminator gene *Q*. In this respect, the genome organization of D3 resembles that of Sfi21-like *Siphoviridae* from low-G+C Gram-positive bacteria¹³.

Regarding the morphogenesis genes, D3 is most closely related to HK022 and HK97, both of which are the well-studied members of the lambdoid group⁴⁶. Bacteriophage HK97 has been shown to possess a unique mechanism of head assembly. Its capsids are assembled from head protein pentamers and hexamers without a scaffold. Instead, HK97 head assembly involves an extended series of transitions, from prohead I, to prohead II, head I, and head II^{19, 20}. In the last transition, head protein subunits are autocatalytically cross-linked, and bridged pentamers and hexamers are associated in a complex catenated structure called "protein chainmail"¹⁸. The head gene organization of D3, which is identical to that of HK97, together with the results of virion protein analysis, suggests that the D3 head assembles in a similar way as in HK97²⁵.

tRNA genes on the D3 genome encode tRNAs specific for Met (AUG), Gly (GGA), Asn (AAC), and Thr (ACA). The translation rate of phage proteins encoded on the relatively AT-rich D3 genome may be influenced by these tRNAs, particularly the glycine and threonine tRNAs, which recognize codons that are rarely used in *P. aeruginosa* but more frequently in D3⁵³. Another interesting point regarding the tRNA genes is that the tRNA genes exhibit homology to similarly functioning tRNA genes derived from Gram-positive

bacteria and their phages, suggesting that the recombinational evolution of D3 may extend outside the γ -subdivision of the Proteobacteria. The large subunit of terminase and the capsid maturation protease of D3 also show sequence similarity to the analogous proteins from the *Siphoviridae* infecting Gram-positive bacteria⁵².

D3 genes responsible for the seroconversion were not fully identified by the initial analysis of the whole genome sequence, only a gene presumably encoding fucosamine O-acetylase⁵². Soon after the publication of the genome sequence, however, Newton *et al.*⁸³ identified three genes required for serotype conversion by the functional analysis of the 3.6-kb fragment located at the 21–24.5-kb region of the D3 genome. These three genes encode an α -polymerase inhibitor (*iap*), an O-acetylase (*oac*), and a β -polymerase (*wzy* β), and form a simple but elegant system by which bacteriophage D3 alters the surface property of *P. aeruginosa*. The *iap* protein inhibits the assembly of the O5 serotype-specific B-band LPS, which is mediated by a chromosome-encoded α -polymerase, and allows the phage-encoded β -polymerase (*Wzy* β) to form a new β -linked B-band LPS. In addition, the D3 O-acetylase (*Oac*) alters the LPS by O-acetylating the C4 position of the FucNAc residues in the O-antigen repeat unit. The change in the glycosidic linkage between O-antigen units, from α to β , represents a change from serotype O5 to serotype O16, and the O-acetylation of FucNAc is characteristic to serotype O20⁸³.

6.3. Large-Size Myovirus ϕ KZ

Bacteriophage ϕ KZ, which has a long contractile tail and a giant icosahedral capsid 120 nm in diameter, is a virulent phage that infects a wide range of *P. aeruginosa* strains^{55, 107}. The ϕ KZ genome is a linear, circularly permuted and terminally redundant double-stranded DNA of 280,334 bp⁷³. It is the largest bacteriophage genome among the published complete sequences (compare the size with that of coliphage T4: 168,903 bp). The average G+C content is 36.8%, which is very similar to that of coliphage T4, but much lower than that of the host *P. aeruginosa* genome (66.6%).

The ϕ KZ genome encodes 306 ORFs and six tRNA genes. The majority of these ϕ KZ ORFs are transcribed in the clockwise orientation (Figure 2). Among the 306 ORFs, only 59 gene products exhibit sequence similarities to known proteins. In most cases, the observed sequence homology is very low. These 59 genes contain several genes involved in nucleotide metabolism, such as genes for dihydrofolate reductase, large and small subunits of ribonucleoside diphosphate reductase, thymidylate synthetase, thymidylate kinase, and deoxycytidine triphosphate deaminase, but no phage-encoded DNA polymerase, DNA-replication associated proteins, and single-stranded DNA-binding proteins were

identified by the sequence homology. These results indicate that ϕ KZ represents an evolutionary distinct branch of the *Myoviridae* family.

Six tRNA genes identified on the ϕ KZ genome encode tRNAs specific for Met (AUG), Asn (AAC), Asp (GAC), Leu (UUA), Thr (ACA), and Pro (CCA). Since the UUA and CCA codons are frequently used in the ϕ KZ ORFs but rarely in *P. aeruginosa* genes, the leucine and proline tRNAs may influence the rate of phage protein translation⁷³.

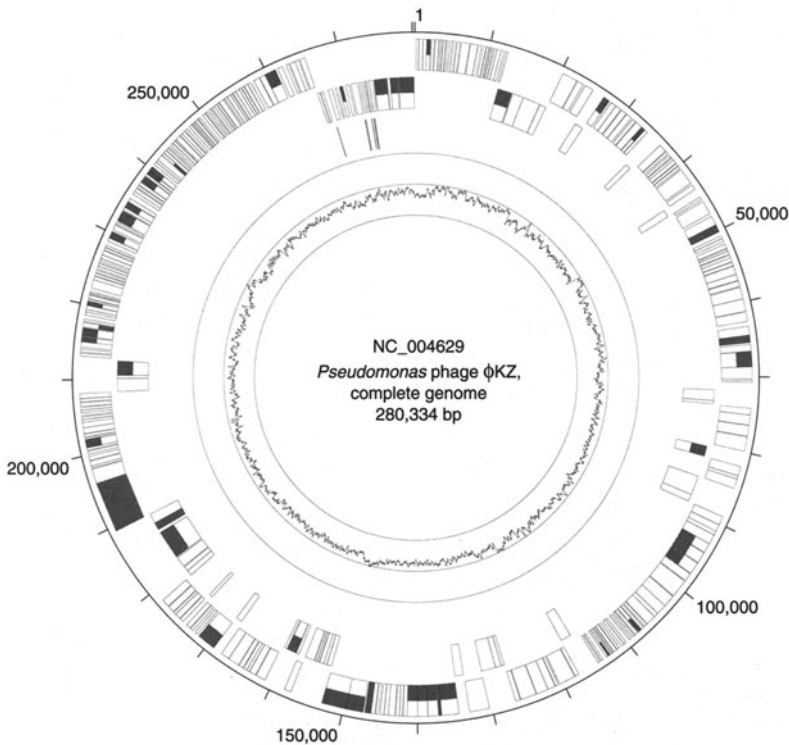


Figure 2. Circular representation of the ϕ KZ genome. The outermost circle indicates the genome position in base pairs. The ϕ KZ ORFs transcribed in the clockwise direction are depicted in the second circle and those in anticlockwise in the third. The locations of six tRNA genes are indicated in the fourth circle. The base composition of the ϕ KZ genome, percentage G+C content plotted by a 300-bp window, is shown in the inner part (three circles each represent 0, 50, and 100 mol% from the innermost one). The ϕ KZ ORFs exhibiting significant homologies to known proteins (E value threshold in BLAST search: 10^{-5}) are indicated by shading the outer parts of ORFs, and those exhibiting significant homologies to its own ϕ KZ ORFs by shading the inner parts. In our analysis using this criterion, ϕ KZ has been shown to contain five paralog groups: Group 1 (PHIKZ056, 072, and 179), Group 2 (PHIKZ093, 094, and 095), Group 3 (PHIKZ131, 132, 133, 134, and 135), Group 4 (PHIKZ144 and 181), and Group 5 (PHIKZ209 and 250).

Because the ϕ KZ phage efficiently infects a wide range of *P. aeruginosa* strains, some consider this phage as an attractive therapeutic agent for the *P. aeruginosa* infection (phage therapy). Several gene products, however, exhibit some sequence homology to proteins from pathogenic bacteria, including apparently virulence-related proteins, such as hemolysin and internalin⁷³. Although the level of observed homology is not so high, functional analyses of these genes may be required before employing the phage as a therapeutic agent.

6.4. T7-like Podovirus gh-1

The highly efficient lytic lifecycle of the T7 phage group is characterized by an early emergence of a single-subunit, rifampicin-resistant phage RNA polymerase that recognizes phage-specific promoters²⁹. Several *Pseudomonas* phages (gh-1, Pssy9220, ϕ PLS27, and ϕ PLS743) belonging to the *Podoviridae* family have been assigned to the T7 group⁸⁸. Among these, the genome sequence of gh-1, which was originally isolated in 1966 from sewage using *P. putida* as the host⁶¹, has been very recently determined⁵¹.

The genome of gh-1 is a 37,359 bp, linear double-stranded DNA with direct terminal repeats (DTRs) of 216 bp. The genome size is slightly smaller than those of so far sequenced T7-like phages, coliphages T7 (39,937 bp) and T3 (38,208 bp), and *Yersinia enterocolitica* phage ϕ Ye03-12 (39,600 bp)^{29, 89, 90}. The average G+C content is 57.4%, considerably higher than those of other T7-like phages (48–51%), but slightly lower than that of the *P. putida* chromosome (61.6%)⁸². The genomic organization of gh-1 is very similar to those of the other sequenced members of the T7 group. As in T3 and ϕ Ye03-12, the gh-1 genome contains regions of high homology to T7 that are interspersed with non-homologous regions that encode regulatory elements and small ORFs of unknown function (Figure 3E). As a result, the gh-1 genome contains 31 genes that are common to the T7 family and additional 12 unique and probably nonessential ORFs. The common genes of gh-1 exhibited average amino acid sequence identity of 50% with T3, 47% with T7, and 41% with ϕ Ye03-12. On the gh-1 genome, two potential host σ^{70} promoters and 10 putative phage promoters were identified. The former are located between the left DTR and the phage RNA polymerase gene in the early region, the location analogous to those of the other T7-like phages. Phage promoters that have a consensus sequence of 23 bp similar to those of T3 and ϕ Ye03-12, are also located at the positions analogous to the other members of the T7 group. The positions of rho-independent transcription terminators are also conserved. These data clearly indicates that gh-1 is a member of the T7 *sensu strictu* group, and that the gene expression of gh-1 is regulated by a system very similar to other members of the T7 group²⁹.

The most striking difference between gh-1 and other T7-like phages is that five genes existing between the left end and gene *I* on the T7 genome are absent

in gh-1. Only two genes among these five T7 genes are of known functions; gene 0.3 encodes an anti-restriction protein and gene 0.7 a protein kinase. The gp0.7, together with gp2, is known to mediate the shutoff of the host RNA polymerase in the T7-infecting cells³⁵, but gene 2 is conserved in gh-1 (Figure 3E).

As for the phage receptor, T3, T7, and ϕ Ye03-12 all use LPS as the host cell receptor^{2, 3, 112}. The receptor for gh-1 has also been demonstrated to be LPS⁵¹. In this regard, it may be important to note that the C-terminal parts of the ph-1 tail fiber protein (gp17), which is responsible for receptor recognition, has a different amino acid sequence (Figure 3E). As mentioned before, a similar finding was obtained from the genomic comparison of ϕ CTX and P2⁸⁰.

7. PROPHAGES IN *P. AERUGINOSA* PAO1

As shown in Figure 3, two bacteriophage-related loci were identified on the *P. aeruginosa* PAO1 chromosome^{13, 81, 102}.

7.1. R/F Pyocin Locus

Of the two bacteriophage-related loci identified on the PAO1 chromosome, the locus existing at the nucleotide position of 672,458–703,477 encodes the R2 and F2 pyocins. *P. aeruginosa* produces three different types of bacteriocins called S-type, R-type, and F-type pyocins^{40, 47}. While S-type pyocins are colicin-like bacteriocins evolutionarily related to E2 group colicins⁴⁸, R- and F-types are morphologically the same as phage tails: Inflexible but contractile phage tails (R-type) or flexible but non-contractile ones (F-type). R pyocins are further classified into five subtypes (R1–R5) by the difference in receptor specificity⁴⁷, which was proposed to be determined by the differences in tail fiber proteins⁸⁶. Several subtypes have been identified also in F pyocins (F1, F2, F3, etc.), and they again differ from each other in receptor specificity^{56, 57}. Several bacteriophages called R or F pyocin-related phages have also been identified, and were demonstrated to be genetically and serologically related to R- or F-type pyocins. They include PS3, PS17, and cytotoxin converting phages ϕ CTX and PS17^{33, 45, 49, 96, 97} as R pyocin-related phages, and KF1⁵⁸ as F pyocin-related phage. Furthermore, headless mutants of phage PS17 were shown to behave like a bacteriocin but with considerably lower activity than the native R pyocins⁹⁸. Thus, R- and F-type pyocins were previously speculated to be the variations of defective phages⁴⁷. However, a somewhat different view on the R and F pyocins has emerged from the sequence analysis of the R/F pyocin locus of PAO1 which produces R2 and F2 pyocins^{81, 102}.

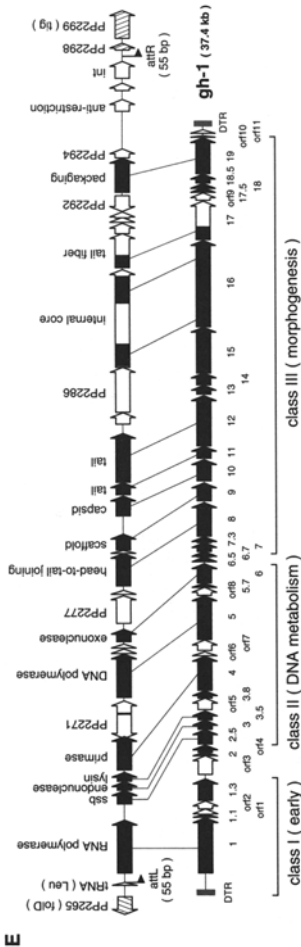


Figure 3. Prophage of *P. aeruginosa* PAO1 and *P. putida* KT2440. A. Locations of prophages and phage-related elements on the chromosomes of *P. aeruginosa* PAO1 and *P. putida* KT2440. B. The gene organization of the R/F pyocin locus on the *P. aeruginosa* PAO1 chromosome. Genes exhibiting sequence homologies to P2-like phage genes are indicated by gray arrows, and those to lambda-like phages by black arrows. Genes showing a sequence similarity to PRF6 are both on P2- and lambda-like phages, and they encode proteins belonging to the TraR family. C. The gene organization of a Pfl-like prophage on the PAO1 chromosome. Genes exhibiting high sequence homologies to Pfl genes are indicated by black arrows. The locations of *attL* and *attR* sites, which we identified, are also indicated. The prophage is integrated into the 3' end of a glycine tRNA gene. In the upstream region of retron-type reverse transcriptase, the TCCCGC sequences, which are repeated 18.5 times in tandem, were identified, but their function is unknown. D. The gene organization of Prophage 2 on the *P. putida* KT2440 chromosome. This locus is annotated as "PYOCIN 01" in the published sequence data, but most likely, this locus is a prophage or phage remnant with a P2-like tail (see the text). Genes exhibiting high sequence homologies to the "PHAGE 03" genes on the chromosome of *Pseudomonas syringae* strain DC3000 (accession no. AE016853) are indicated by gray arrows. For comparison, the names of homologous pyocin genes of *P. aeruginosa* are indicated below each Prophage 2 gene. Genes encoding the LysE amino acid transporter family proteins are indicated by black arrows. E. The gene organization of a T7-like prophage (Prophage 3) on the KT2440 chromosome. For comparison, the genome of *P. putida* T7-like bacteriophage gh-1 is shown, and genes exhibiting sequence homologies are connected by lines. Genes exhibiting sequence homologies to T7 genes are indicated by black arrows. The locations of *attL* and *attR* sites are also indicated. Prophage 3 is integrated into the 3' end of a leucine tRNA gene. Gray boxes at the both ends of the gh-1 genome represent DTRs.

The R2 and F2 pyocin gene clusters are located between *trpE* and *trpG* as demonstrated by Shinomiya *et al.*^{99, 100}. At the most upstream region, the two regulatory genes, *prrR* and *prrN*, which encode a regulatory system resembling that of temperate phages⁷¹, are located, and are transcribed into the opposite direction from other genes. Other genes in this locus are divided into three clusters: A gene cluster of unknown function, the R2 pyocin gene cluster, which is apparently inserted into the lysis gene cassette, and the F2 pyocin gene cluster (Figure 3B). The R2 and F2 pyocin gene clusters contain nearly complete sets of P2 and lambda tail gene homologues in almost the same order as they are found in those phages. No other phage genes required for head formation, replication, and integration, are contained. Genes responsible for head-tail joining are also missing from both pyocin gene clusters. These genomic features imply not only that each pyocin is derived from a common ancestor with the P2 or lambda phage family, but also that both types of pyocins are phage tails that have been evolutionarily specialized as bacteriocins⁸¹. In fact, both pyocins share a single lysis system and a single gene regulatory system. Moreover, gene expression of S-type pyocins is also controlled by the *prrRN* regulatory system, and is coordinately regulated with R- and F-type pyocins⁷¹.

In other *P. aeruginosa* strains known to produce different subtypes of R and F pyocins, R/F pyocin loci have been demonstrated to be present also between *trpE* and *trpG* by the PCR scanning analysis⁸¹. The results also indicated that the gene organizations of other subtypes are principally the same as that of the R2/F2 locus of PAO1, but the genes for tail fiber formation, especially the C-terminal portion of tail fiber, which is responsible for the receptor binding, are altered. These differences probably correspond to the different killing spectra of each subtype of R or F pyocin. Furthermore, in *P. aeruginosa* strains producing only F- or R-type pyocin, DNA segments encoding the R or the F pyocin genes are completely deleted by being replaced by short DNA segments. These notions are in good agreement with the recently published data obtained by DNA microarray analysis of 18 *P. aeruginosa* strains from various clinical and environmental sources¹¹³. All the examined strains contained R and/or F pyocin gene clusters, but the tail fiber genes of both types of pyocins were highly variable, making a sharp contrast to the fact that regulation and lysis genes shared by both pyocins were invariably present in all strains.

In the unpublished genome sequence of a *P. syringae* strain DC3000 (accession no. AE016853), a lambda-like tail gene cluster is also located between *trpE* and *trpG*. Since no head gene is found in this locus, we can consider that this locus also encodes an F pyocin-like bacteriocin. However, unlike the *P. aeruginosa* R/F pyocin locus, the *P. syringae* locus contains two

integrase-like genes and a colicin M-like gene. In addition, several genes unrelated to phage function, such as the genes for an autotransporting lipase, type III effectors HopPtoH and HopPtoC, and an ImpB/MucB/SamB family protein, are presented outside of the prophage-like region but between the *trpE* and *trpG* genes. Thus, it may be better to consider this locus as a prophage remnant.

7.2. Pfl-like Prophage

The second phage-related element on the PAO1 chromosome is a Pfl-like prophage located at a genome position of about 789–796 kb. This locus contains 11 genes that are highly homologous to the genes of a filamentous phage, Pfl, with a completely conserved gene organization (Figure 3C). These genes are flanked upstream by genes for retron-type reverse transcriptase and an ATP-binding protein (PA0715 and 0716) and downstream by an integrase gene (PA0728). The exact prophage–chromosome junction was not identified in the original publication. However, by sequence inspection of the upstream region of PA0715 and the downstream region of PA0728, we have identified possible *attL* and *attR* sequences of 27 bp (5'-TGGAGCGGGC-GAAGGGAATCGAACCCT-3') that correspond to the 3' part of the glycine tRNA genes located downstream of a putative plasmid stabilization gene (PA0729). Thus, the Pfl-like phage is integrated into the tRNA gene, and it is likely to have carried the genes for reverse transcriptase, an ATP-binding protein, and a plasmid stabilization protein into the PAO1. The presence of an integrase gene in this prophage is of particular interest because filamentous phages usually do not integrate into the chromosome but exist in the cytosol as double-stranded RF forms like plasmids. Although two filamentous phages from *Vibrio cholerae* (CTX ϕ) and *V. parahaemolyticus* (f237) are known to be integrated into the *dif* site in the replication termination region of the their host chromosomes^{44, 111}, the integration is mediated by bacterial enzymes, XerCD⁴².

Data from the abovementioned DNA microarray analysis indicated that this locus is one of the most variable regions on the *P. aeruginosa* chromosome¹¹³. In particular, genes for reverse transcriptase and an ATP-binding protein are present only in PAO1. The plasmid stabilization gene is present in seven strains including PAO1. However, only four out of the examined 18 strains completely lack this locus, and other strains contain 6–12 genes of this Pfl-like prophage. These data suggest that Pfl-like phages are widely distributed in *P. aeruginosa*, but their genomes are highly diverged. Thus, they seem to have played some role in generating the genomic diversity in *P. aeruginosa* strains.

8. PROPHAGES IN *P. PUTIDA* KT2440

The sequenced *P. putida* strain, KT2440 contains four prophages, though their genomic features have not yet been analyzed in detail^{13, 14, 82}.

8.1. Prophage 1

Prophage 1, which encodes 72 ORFs (PP3849-PP3920), is located at a nucleotide position of 4,371,606–4,427,563, and is integrated into a cysteine tRNA gene. This phage is a hybrid of an Sfi21-like siphovirus head gene cluster and a Mu-like myovirus tail gene cluster. The closest match for the head genes is found in the *Burkholderia* siphovirus ϕ E125¹¹⁴, and the tail genes in the *Shigella* phage SfV though a group II intron is inserted between the tail sheath and the tail tube genes (PP3869 and PP3867). This prophage seems to have carried several “lysogenic conversion genes” into KT2440; a gene for hemolysin-type calcium-binding protein (PP3848), homologues of the *hicAB* genes that have been found in the major pilus gene cluster of *Haemophilus influenzae* (PP3899 and PP3900), and a cytosine methyltransferase gene (PP3912).

8.2. Prophage 2

Prophage 2 is located at the nucleotide position of about 3,418–3,447 Kb. This locus is annotated as “PYOCIN 01” in the published genome sequence data. Actually, this region contains a nearly complete set of R pyocin gene-homologues (Figures 3B and D). This locus, however, also contains gene encoding proteins required for phage head formation, such as a large subunit of terminase, portal protein, and head maturation protease, between the holin gene (PP3040) and the tail gene cluster (PP3056-PP3066) that are highly homologous to the R pyocin genes of *P. aeruginosa* (Figure 3D). Thus, this locus should be considered as a P2-like prophage or its remnant rather than the R-type pyocin locus. *P. syringae* strain DC3000 (accession no. AE016853) contains a prophage (PHAGE03; PSPTO3385-3429) that is more closely related to this prophage than R pyocin. As many as 31 genes on this locus, including the R pyocin gene-homologues and an cytosine methyltransferase gene (PP3029), exhibit high sequence homology to those on PHAGE03 of *P. syringae* (genes depicted by gray arrows in Figure 3D). Furthermore, their gene arrangement is completely conserved. We could not identify the *att* sites for Prophage 3, but since PP3027 is a homologue of a ϕ CTX early gene and homologues of PP3026 have been identified on many bacteriophage genomes (note that, although some are annotated as integrase, their functions have not yet been determined), this prophage region encompasses at least from

PP3026 to PP3066. It is possible that this locus has undergone some genomic rearrangement, and thus the original structure of chromosome–prophage junctions has been lost. In fact, the region upstream of this locus contains three genes encoding LysE amino acid transporter family proteins homologous to each other (PP3021, 3023, and 3025), but the second gene seems to have been split into two and the 3' terminal part translocated to the other side of the prophage region (PP3068).

8.3. Prophage 3

Prophage 3 is located at the nucleotide position of 2,586,037–2,626,082, and integrated into a leucine tRNA gene. The genome of this phage, which encodes 32 ORFs (PP2266–PP2297), is very similar to that of the T7-like *Podoviridae*; the majority of closest matches is found in the T7 group, and the gene organization also closely resembles that of the T7-like phages (Figure 3E). This finding is very surprising because all the T7-like phages known so far are lytic phages (see the section of gh-1 in this chapter). Two Class I early genes, the phage RNA polymerase and anti-restriction genes (PP2266 and PP2295, note that gh-1 lacks the anti-restriction gene while other T7-like phages contain the gene in the upstream region of the RNA polymerase gene), are each located at the left and right end of the prophage genome, and the integrase gene, which is absent in the T7 group, is located at the right end (PP2297). Thus, this phage seems to have evolved from a T7-like phage by capturing a gene cassette for lysogenization in its Class I region, and would be a very interesting research material to study the mechanism of phage evolution. A DNA ligase gene (gene *l.3*) shared by the T7 group is missing from this prophage, and no sequence similar to the DTR of gh-1 is present, suggesting that the structure of genome terminus has also been altered in this phage during the evolution from a lytic phage to a temperate phage. Furthermore, although all the genes on the lytic T7-like phages are transcribed into one direction, and indeed no lysogenic conversion gene is encoded, this prophage contains two genes transcribed into the opposite direction from others. Functions of both genes are not known, but they are likely to be the “lysogenic conversion genes,” which this phage has acquired after it was converted to a temperate phage.

8.4. Prophage 4

Prophage 4 is located at the genome position of 1,737,979–1,777,414, and integrated into the 5' end of an ORF of unknown function (PP1531). The genomic organization and the amino acid sequence of each gene product of the structural genes are highly homologous to those of the *P. aeruginosa* phage

D3, suggesting that this phage is a lambda-like prophage. The genome size (39.4 kb) is much smaller than D3 but similar to well-known lambda-like coliphages HK97 and HK022 (Figure 1). The genes for head formation are similar to those of HK97 as well. This phage also contains two methylases (PP1535 and 1541), which may be "lysogenic conversion genes." A curious feature of this phage is that, in addition to the true integrase of this phage (PP1532), another gene homologous to phage integrases (PP1555) is present in the early region, between replication genes and an anti-terminator gene. The function and biological significance of this second integrase is not known, but Prophage 1 also contains a second integrase-like gene (PP3889) at the analogous position to that on Prophage 4, and both second integrases are highly homologous, exhibiting more than 50% amino acid sequence identity.

9. CONCLUSIONS

In the last five years, we have obtained a large amount of genome sequence information on *Pseudomonas* phages. These sequence data have considerably expanded our knowledge on the biology, genetics, and evolution of *Pseudomonas* phages. As has been demonstrated in ϕ CTX, D3, and gh-1, the genomic comparison with well-studied coliphages, such as P2, lambda, and T7 phages, revealed that *Pseudomonas* phages often share common ancestors with these coliphages, and by the analogy to these coliphages, we could obtain deeper understanding of the biological features of these *Pseudomonas* phages. More striking findings were obtained by the genome analysis of R/F pyocin locus on the *P. aeruginosa* chromosome: It is now clear that these phage tail-like bacteriocins are indeed phage tails that have evolved specifically as bacteriocins from common ancestors with P2 or lambda phages. The genomic comparison of these phages also reveals how their genomes have diverged during their intergeneric evolution, providing evidence to show that highly complicated and extensive recombination have played important roles in the evolution and diversification of bacteriophages. It is also likely that bacteriophages have played significant roles in generating the intraspecific genomic diversity of *Pseudomonas*. The sequenced *P. aeruginosa* strain PAO1 and *P. putida* strain KT2440 contain two and four prophages, respectively. The unpublished genome sequence of *P. syringae* strain DC3000 also contains at least six prophages. These phages seem to have carried considerable numbers of "lysogenic conversion genes" into each strain. As demonstrated in *P. aeruginosa*, these prophage loci are probably highly variable in each species. What we should emphasize at the end is that so many different types of bacteriophages were identified in *Pseudomonas*, far more than in many other bacterial genera. In the next decade, we are sure to obtain more phage

genome sequences. It is quite promising that, using these data, we will be able to illustrate more precisely how such a huge variety of bacteriophages are evolving and spreading in *Pseudomonas*, and how they are contributing to the genomic and phenotypic diversification in *Pseudomonas* strains.

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TRANSPOSITION AND OTHER MUTATIONAL PROCESSES IN *PSEUDOMONAS*

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1. INTRODUCTION

The genus *Pseudomonas* represents the most diverse and ecologically widely distributed group of bacteria²¹⁴. Members of this genus are able to colonize many different environments, including soil and water, but also plant and animal tissues. Diversity arises and is maintained through interplay between ecological and genetic factors. Genetic variation is generated by point mutations and recombination events. Transposition is a recombination reaction that mediates the movement of discrete DNA segments between nonhomologous sites. Insertion of a transposable element into a gene usually inactivates it. However, many transposable elements have been shown to activate the expression of neighbouring genes³⁰. Multiple copies of the element can also act as substrates for homologous recombination that can lead to deletions, inversions, duplications or more complex alterations in the genome structure. Also, horizontally transferred genes can be frequently disseminated among bacterial populations as components of mobile genetic elements. The aim of this chapter is to give an overview of mobile DNA elements described in *Pseudomonas* species focusing on the mechanisms of transposon-associated genetic variation. The mechanisms of other mutational processes occurring in pseudomonads is also discussed.

2. TRANSPOSABLE ELEMENTS IN BACTERIA

Organisation of different transposable elements in bacteria is presented in Figure 1. The simplest bacterial transposable elements are insertion sequences (IS). Features and properties of IS elements are extensively

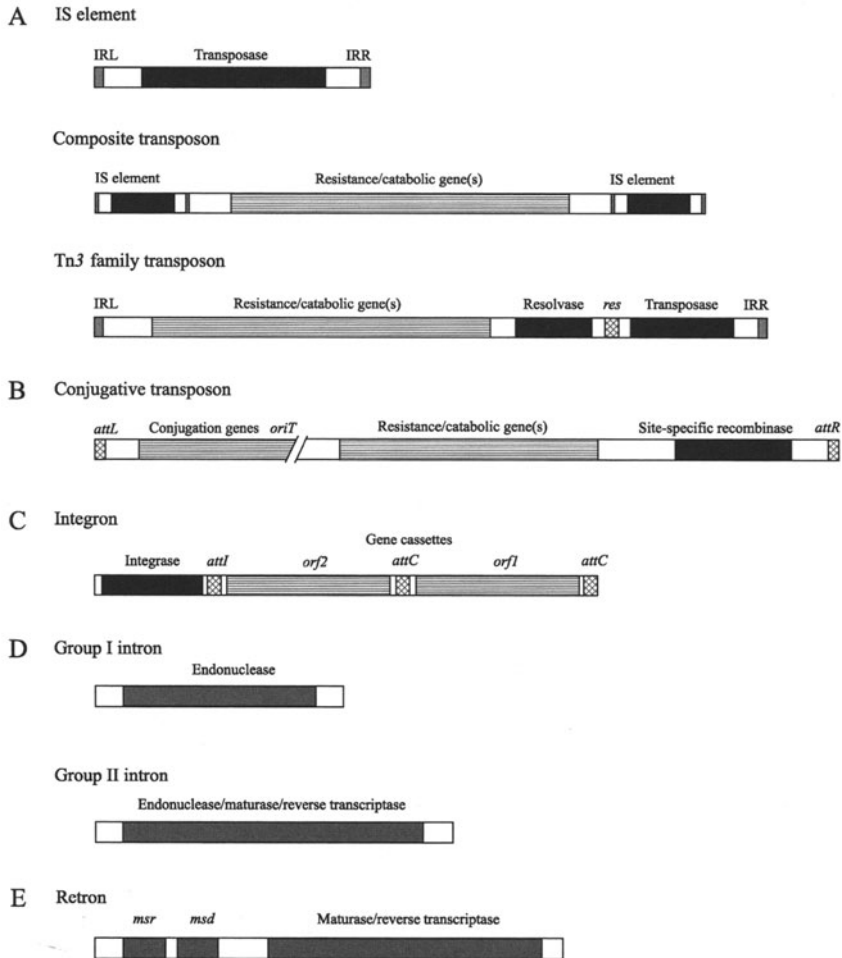


Figure 1. Organisation of mobile elements in bacteria. A. IS elements and transposons. The terminal inverted repeats IRs that are binding sites for transposase are marked as grey boxes (IRL and IRR). IS elements³⁰ are the simplest transposable elements. Two copies of the same IS element can form a composite transposon. Tn3 family transposons⁷⁴ usually transpose by a replicative pathway forming an intermediate, called cointegrate. A site-specific recombination system

reviewed³⁰. IS elements are small and genetically compact. Generally they encode no other functions than those involved in their mobility. The majority of IS elements contain short inverted repeat sequences (IRs) ranging usually between 10 and 40 bp. IRs are specifically recognised and bound by an element-encoded transposase. IS elements like many other mobile DNA elements are flanked by directly repeated sequence (DR) of the target DNA. Target site duplications arise from staggered cleavage of the double-stranded DNA molecule. A given element will generally generate a DR of defined length. As a result of efforts in bacterial genome and plasmids sequencing, the list of IS elements is growing rapidly (see relevant database file at <http://www-IS.biotoul.fr>). According to their genetic organisation, similarities in their transposases, IRs, and generation of a DR of determined length, IS elements are classified into 19 families³⁰. Members of most individual families are distributed across many different eubacterial genera.

IS elements are able to form composite transposons. Well-known examples of composite transposons include a pair of IS elements flanking a segment of DNA, containing one or more genes encoding for functions like resistance to either antibiotics or heavy metals or catabolism of organic compounds. The transposition of a composite transposon is catalysed by the transposase encoded by one or both copies of the IS element. There are many IS elements (and composite transposons) that transpose as simple insertions.

Figure 1. Continued

(serine recombinase of the resolvase/DNA invertase family or tyrosine recombinase of the lambda integrase family) promotes the conversion of cointegrate into two separate components—the target with a simple insertion of the element and the regenerated donor replicon. The cointegrate resolution takes place at a *res* site (shown as crosshatched box in the figure). Transposase and site-specific recombinase-encoding regions are indicated by black boxes. The striped boxes designate regions encoding other functions not related to transposition. B. Most conjugative transposons³⁴ encode site-specific recombinases that belong to the lambda integrase family (black box in the figure). Conjugative transposons move via circular intermediate that is produced by excision of the integrated element from the donor chromosome. Some elements require for excision a specific protein analogous to lambda Xis protein. Excision and circularisation result in a closed junction (*attP*) between left (*attL*) and right (*attR*) ends of the element. This junction (*attP*) recombines with a target site (*attB*) in the chromosome or on large plasmids. The circular form can transfer by conjugation to a new recipient cell in which reintegration can take place. C. Integrons¹⁸³ are the recombinase-encoding genetic elements into which gene cassettes are integrated and excised. Gene cassettes contain a recombination site designated *attC* and antibiotic resistance or other genes (striped boxes). Incoming cassettes are preferentially integrated at the *attI* site. D. Group I intron mobility depends on an intron-encoded site-specific DNA endonuclease (grey box in the figure). The movement of a group II intron involves an RNA intermediate. In addition to the endonuclease activity, the group II intron-encoded proteins have maturase and reverse transcriptase activities¹⁰. E. Retrons²⁶² consist of three genes (indicated by grey box): *msr* for the RNA-coding region, *msd* for the msDNA-encoding region, and the reverse transcriptase-encoding region.

The Tn3 family transposons, in contrast to composite transposons, consist of a single transposable segment. They have similar IRs, generally of about 40 bp, and their transposition usually generates a 5-bp duplication of the target DNA. Like composite transposons, Tn3 family transposons include additional genes not related to transposition (e.g., resistance genes or catabolic operons). The Tn3 family transposons transpose using a replicative mechanism. However, some elements are capable of both replicative and conservative transposition. The organisation of transposons and transposition mechanisms of Tn3 family were recently reviewed⁷⁴.

Conjugative transposons are characterised by their ability to transfer laterally using a process that requires intercellular contact (see ref. [34] for a recent review). Conjugative transposons can excise from the chromosome, form a circular intermediate, and enter a new cell via conjugation. Unlike plasmids, the circular intermediate does not appear to replicate. Once in the recipient, the circular intermediate integrates into the recipient genome. Excision and integration mechanisms of conjugative transposons are similar to reactions catalysed by site-specific recombinases of temperate bacteriophages. Some conjugative transposons (e.g., Tn916) integrate into multiple sites in the genome of the recipient. Other elements utilise only a single target³⁴. Structural genes for tRNAs are common targets for insertions of bacteriophages and some conjugative transposons. Integrating genetic elements that use tRNA genes as targets create a duplication, restoring the tRNA gene²⁸.

Integrans are genetic elements that encode a site-specific recombination system which recognises and captures mobile gene cassettes (see ref. [183] for a recent review). A minimal integran consists of an integrase gene *intI* and an adjacent recombination site (*attI*). Integrans contain one or more non-autonomous mobile gene cassettes integrated at *attI*. The insertion of a gene cassette takes place by integrase-mediated site-specific recombination between the *attC* site of the circularised cassette and the *attI* site of the integran. Gene cassettes can be assembled into tandem arrays within the integrans. Many integrans, in turn, are located within transposons typically residing on broad host range conjugative plasmids, and are thereby frequently disseminated throughout a bacterial population by horizontal transfer.

Genomic islands are chromosomal gene clusters that appear to be derived from mobile sequences, although most of them are no longer mobile. They have no obvious characteristics other than those indicative of horizontally transferred loci. Genomic islands are found as compact, distinct units with a G+C content different from the remainder of the chromosomal DNA of the host. For recent reviews on genomic islands, see ref. [42] and [81]. The DNA flanking a genomic island often contains remnants of viral and transposase genes, like traces marking the way of acquisition of this genetic material. They are often large (30–100 kb), however, “microislands” made of one or a

few genes have been described as well. Genomic islands are sporadically present in some strains and absent in other strains within the same or related species. Depending on the functions that are encoded by genomic islands, they may be called pathogenicity islands (PAIs), symbiosis islands, or metabolic islands.

Prokaryotes contain reverse transcriptase-encoding retroelements that fall into two basic types: Group II introns that use reverse transcriptase (RT) to mobilise the intron element to new locations (discussed below), and retrons²⁶². Retrons encode RT and multicopy single-stranded DNA (msDNA). msDNA molecules consist of a short single-stranded DNA covalently linked to an internal G residue of the RNA molecule by a 2',5'-phosphodiester linkage. Initiation of cDNA synthesis begins at the 2'-OH group of the branching G residue, followed by cDNA synthesis using the same RNA transcript as a template. Although the function of msDNA is unknown, it has been hypothesised that retrons may be associated with bacterial pathogenicity because of their role in the enhancement of mutation rate in pathogenic bacteria during the host invasion^{125, 262}.

Group I and group II mobile introns are found in eubacteria and in eukaryotic organelles that have evolved from eubacteria (for a review, see refs [10] and [133]). It has been hypothesized that group II introns have migrated to the nucleus of eukaryotic cell, where they have evolved into spliceosomal introns as well as non-LTR retrotransposons and telomerase¹⁰. Group I introns do not interrupt protein-coding genes in bacterial chromosomes (they interrupt only tRNA genes) in contrast to phage-encoded group I introns that always interrupt protein-coding genes, many of which are involved in some aspect of DNA metabolism¹³³. Most of the bacterial group II introns identified to date, interrupt known or putative IS elements and transposons and this is suggested to favour the spread of the introns within and between species^{54, 133}. Group I and II introns are capable of inserting into the same site in a cognate intronless allele (intron homing) or into novel genomic sites (intron transposition). Intron transposition, relative to intron homing, is an infrequent process due to the reduced affinity of intron-encoded proteins for variants of their recognition sequences and to heterology between intron-containing and intronless alleles⁵⁴.

3. TRANSPOSABLE ELEMENTS IN *PSEUDOMONAS*

Most of the families of IS elements classified by Chandler and Mahillon³⁰ are represented among natural isolates of *Pseudomonas* strains (see <http://www-IS.biotoul.fr>), whereas members of the IS5 family are most frequently recovered. There is also a number of IS elements characterised belonging to the IS3, IS21, and ISL3 families. Several members of IS elements described in *Pseudomonas* are associated with composite transposons.

Notably, many catabolic transposons described in *Pseudomonas* are composite transposons²³¹. However, the best-characterised catabolic operons in *Pseudomonas*, the toluene-degrading (*xyl*) genes on plasmid pWW0, are encompassed by Tn3 family transposons²⁴⁰. Also, many transposons carrying genes for mercury-resistance belong to Tn3 family^{74, 101, 145}. Other mobile elements like conjugative transposons, integrons, class I and II introns, and gene islands are also represented in pseudomonads. Integrons, in turn, are frequently found within the Tn3 family transposons. Most of the composite catabolic transposons do not carry all the genes necessary for conversion of the initial substrates to the central metabolites. This contrasts with the Tn3 family catabolic transposons and the degradation islands.

3.1 Transposable DNA Elements in the Genomes of *P. aeruginosa* PAO1 and *P. putida* KT2440

Search in the IS elements database at <http://www-IS.biotoul.fr> shows that the completely sequenced genome of *P. aeruginosa* PAO1 contains one copy of an IS110 family element and two distinct IS3 family elements, one of which is present in five copies and the other in a single copy. Also, 10 regions of PAO1 genome with sizes from 3 to 21 kb exhibited a significantly lower G+C content and unusual codon usage, possibly indicative of recent horizontal gene transfer²²¹. There is also possibility that some genetic material has been acquired from an eukaryotic organism²⁵⁷.

Analysis of the complete genome sequence of *P. putida* KT2440¹⁵³ has revealed the presence of seven novel multicopy IS elements, a Tn7-like element, and Tn4652 that was originally found in TOL plasmid pWW0²⁴². These elements are listed in Table 1. Genome analysis of *P. putida* KT2440 has indicated that mobile elements have played a significant role in the acquisition of metabolic pathways. Around 80% of the chromosome shares a similar G+C content and oligonucleotide bias, whereas 105 islands showed atypical G+C content and/or oligonucleotide signature²⁵⁵. Twenty-nine of these islands carry a signature of mobile elements such as phages, transposons, IS elements, and group II introns, indicating a recent acquisition by horizontal gene transfer²⁵⁵.

3.2. IS elements and Composite Transposons in Natural Isolates of *Pseudomonas*

3.2.1. Transposable Elements in Different Xenobiotic-Degrading Pseudomonads. Most IS elements associated with catabolic genes in *Pseudomonas* are the members of either the IS5 or the ISL3 family (Table 2). Additionally, there is

Table 1. Transposable elements in the genome of *P. putida* KT2440.

Element	Family	Copy number
IS <i>Ppu8</i>	IS4	6
IS <i>Ppu9</i> ^a	IS110	9 (2 truncated)
IS <i>Ppu10</i> ^a	IS110	7
IS <i>Ppu11</i>	IS110	2
IS <i>Ppu13</i>	IS66	2
IS <i>Ppu14</i>	IS66	6
IS <i>Ppu15</i>	IS66	5
Tn4652 ^b	Tn3	1
IS1246 ^c	IS5	1
Tn7-like element		1
Group II intron ^d		8

^aThe localisation of IS*Ppu9* and IS*Ppu10* in the chromosome of KT2440 indicates that these elements selectively target *P. putida*-specific repetitive extragenic palindromic (REP) sequences¹⁵³.

^bTn4652 has transposed into the bacterial chromosome from TOL plasmid pWW0 during pWW0 elimination. This transposon is a derivative of Tn4651 that has a deletion of catabolic region including *xyl* genes as a result of reciprocal recombination between the two copies of IS1246^{184, 242}.

^cLocates within Tn4652.

^dAlmost identical copies exhibiting a high homology to group II intron Xln6 found in *P. alcaligenes* NCIB 9867²⁶⁹.

one prominent IS element, IS1071, that formally belongs to the Tn3 family but lacks a cointegrate resolution system³⁰. Notably, many IS elements listed in Table 2 and below have been identified only by their structural features.

IS1071 was originally identified as an element flanking chlorobenzoate degradation genes (*cba* genes) within the composite transposon Tn5271 in a plasmid of *Comamonas testosteroni* strain BR60¹⁵⁰. This IS element has unusually long, 110-bp IRs. There is no significant homology of the IR sequence extending from 39 to 110-bp with sequences flanking the 38-bp IRs of the Tn3 transposons. Nakatsu *et al.*¹⁵⁰ have proposed that IS1071 may represent an ancestral element from which the Tn3 family has evolved by reducing the IRs and acquiring the site-specific recombination systems (resolvase or integrase), resistance determinants, or catabolic genes. IS1071 has been found to flank the different chlorobenzoate catabolic genes (*clc*, *cba*, or *fsb*) in bacteria, and in many cases it forms a composite transposon^{47, 168}. Additionally, this element is linked to a diverse collection of other catabolic genes (Table 2).

IS5-family elements are widely distributed among *Pseudomonas* strains and like IS1071, they flank a variety of catabolic genes (Table 2). ISL3 family elements can form composite transposons, as shown in benzene-degrading *P. putida* strain ML2 in which two copies of IS1489 have formed composite transposon Tn5542⁵⁸. However, most of the ISL3 family elements discovered in *Pseudomonas* have been identified due to insertions that have activated or inactivated certain catabolic genes (Table 2). For example, IS*Ppu12*, which

Table 2. Association of IS elements with catabolic genes in independent *Pseudomonas* isolates.

IS element	Family	Host (location on plasmid)	Growth substrate (genes)	Comments	References
IS1071 ^a	Tn3	<i>P. putida</i> UCC22 (pTDN1) <i>Pseudomonas</i> sp. ADP1 (pADP-1) <i>P. pseudocataligenes</i> POB310 <i>P. pavonaceae</i> 170	Aromatic amines (<i>tdnQTA, 4,5BR</i>) Atrazine (<i>atzA, atzB, atzC</i>) Carboxydiphenyl ethers (<i>pobAB</i>) 1,3-dichloropropene (<i>dhaA</i>)	Flanks <i>tdn</i> genes at both sides Flanks each <i>atz</i> gene at both sides Upstream of <i>pobAB</i> genes Multiple copies around <i>dhaA</i>	[65] [132] [45] [171]
IS1246	IS5	<i>P. putida</i> mt-2 (pWW0) <i>P. putida</i> TMB	Toluene and xylenes (<i>xyl</i> operons) Xylenes and methyl-benzenes (<i>imb</i>)	Flanks <i>xyl</i> operons at both sides in Tn4651 Locates in <i>imbU</i> and <i>imbR</i> spacer region	[184] [56]
IS1472	IS5	<i>Pseudomonas</i> sp. EST1001 <i>P. putida</i> 01G3	Phenol (<i>pheBA</i>) Alkylbenzene (<i>ebd</i> operon)	Locates between <i>pheBA</i> genes and their promoter Upstream of <i>ebdAa</i> gene	[93] [29]
IS5-like element ^{b,c}		<i>P. stutzeri</i> AN10	Naphthalene (<i>nah</i> operons)	<i>mpA1</i> downstream of <i>nahD</i> gene	[17]
IS5-like element ^b		<i>P. stutzeri</i> AN10	Naphthalene (<i>nah</i> operons)	<i>mpA2</i> downstream of <i>nahW</i> gene	[18]
IS5-like element ^b		<i>P. stutzeri</i> AN10	Naphthalene (<i>nah</i> operons)	<i>mpA3</i> upstream of <i>nahW</i> gene	[18]
IS5 ^{car1}	IS5	<i>Pseudomonas</i> sp. CA10	Carbazole (<i>car</i> and <i>ant</i> genes)	Upstream of <i>car</i> operon, identical to IS ^{car4}	[156]
IS5 ^{car2}	IS5	<i>Pseudomonas</i> sp. CA10	Carbazole (<i>car</i> and <i>ant</i> genes)	Upstream of <i>antABC</i> , differs from IS5 ^{car2} by 1 nt	[156]
IS5 ^{car3} ^d	IS5	<i>Pseudomonas</i> sp. CA10	Carbazole (<i>car</i> and <i>ant</i> genes)	Downstream of <i>antABC</i>	[156]
IS5 ^{car4}	IS5	<i>Pseudomonas</i> sp. CA10	Carbazole (<i>car</i> and <i>ant</i> genes)	Downstream of <i>car</i> operon	[156]
IS1489v1	ISL3	<i>P. putida</i> ML2 (pHMT112)	Benzene (<i>bed</i> genes)	Upstream of <i>bed</i> genes, composite Tn (Tn5542)	[58]
IS1489v2	ISL3	<i>P. putida</i> ML2 (pHMT112)	Chlorobenzoates (<i>ohb</i> genes)	Downstream of <i>ohbAB</i> genes	[58]
IS element ^e	ISL3	<i>P. aeruginosa</i> 142	Chlorobenzoate (<i>ohb</i> genes)	Upstream of <i>ohbAB</i> genes	[239]
IS element ^f	ISL3	<i>P. stutzeri</i> OX1	Xylenes (<i>lou</i> and <i>xyl</i> genes)	Switches <i>lou</i> and <i>xyl</i> genes on and off	[239]
ISPs1 ^g	ISL3	<i>P. putida</i> mt-2 (pWW0)	Toluene and xylenes (<i>xyl</i> operons)	Inside Tn4653, insertional inactivation of <i>xyl</i> genes	[15]
ISFpu12	ISL3	<i>P. putida</i> PP3	Dichloropropionic acid (<i>deh</i> genes)	Composite Tn (<i>DEH</i> element), <i>deh</i> genes silencing	[260]
IS1411 ^h	ISL3	<i>Pseudomonas</i> sp. EST1001	Phenol (<i>pheBA</i>)	Downstream of <i>pheBA</i> operon; activates silent genes	[254]
ISFpu1	IS630	<i>P. putida</i> (oleovorans) GPo1	Alkanes (<i>alk</i> genes)	Locates between <i>alkI</i> and <i>alkN</i> genes	[93]
ISFpu2	IS630	<i>P. putida</i> P1	Alkanes (<i>alk</i> genes)	Locates between <i>alkS</i> and <i>alkB</i>	[247]
ISFpu3	IS630	<i>P. putida</i> P1	Alkanes (<i>alk</i> genes)	Locates between <i>alkS</i> and <i>alkB</i> , flanks ISFpu2	[247]
ISFpu4	IS3	<i>P. putida</i> P1	Alkanes (<i>alk</i> genes)	Composite transposon TnFpu-alkI	[247]

ISPpu5	IS110	<i>P. putida</i> P1	Alkanes (<i>alk</i> genes)	Downstream of <i>alkT</i> , ISPpu4 insertion in left end	[247]
IS1066	IS630	<i>Pseudomonas</i> sp. P51	Chlorobenzene (<i>tcbAB</i>)	Forms composite Tn with iso-IS element IS1067	[249]
IS element ⁱ	IS21	<i>Pseudomonas</i> sp. P51	Chlorocatechols (<i>tcbR-CDEF</i>)	Remnants of IS element flank <i>tcb</i> cluster at both sides	[161]

^aThis IS element is associated with variety of aromatic or aliphatic components degradation genes in other bacteria (e.g., degradation of chlorobenzoates in *Comamonas testosteroni* BR60¹⁵⁰ and CPE3⁴⁷; chloroacetate in *Delftia acidovorans* B⁹⁸; o-phthalate in *Comamonas acidovorans* UCC61⁵²; carboxydiphenyl ethers in *P. pseudodolcaligenes* 2a²⁶¹ and in *Achromobacter xylosoxidans* subsp. *denitrificans* strain EST4002²⁵⁰; p-sulfobenzozate in *Comamonas testosteroni* T-2⁹²; aniline in *Comamonadaceae*¹⁶).

^bClassified as IS5-like element by *tnpA* sequence analysis.

^cTnpA sequence (designated as TnpA3) 95% identical to TnpA of IS1384⁴⁸.

^dTnpA sequence 98% identical to *tnpA1* found downstream of *nahD* gene in *P. stutzeri* AN10¹⁷.

^eIS1396-like element, 99% identical to IS1396¹¹¹.

^fIS1396-like element, 94% identical to IS1396-like element in *P. aeruginosa* 142²³⁹.

^gIS1396-related element, TnpA 93% identical to TnpA of IS1396.

^hIS1411 *tnpA* gene identical to the *tnpA* downstream of the chlorobiphenyl degradation genes *bph* in *Burkholderia cepacia* strain JHR22. Traces of IS1411 have also been found in the nitrotoluene-degrading *Pseudomonas* sp. strain TW3⁹⁰.

ⁱIS1600-related element; IS1600 forms composite transposon Tn3707 in *Ralstonia eutropha* chlorocatechol-degrading strain NH91¹⁶¹.

originally lies on pWW0 in a region adjacent to the right end of Tn4651 (inside the right arm of Tn4653), can generate catabolic mutants by insertion into *xyl* genes on pWW0²⁶⁰. Copies of IS*Ppu12* flank on either side the gene of hydrolytic dehalogenase (*dehI* gene) and its cognate regulatory gene *dehR_I* forming a composite transposon *DEH* able to transpose among a broad range of bacteria²⁵⁴. The transposition frequency of the *DEH* element is unusually high, and transposition of either the composite transposon or IS*Ppu12* alone has shown to be associated with genomic rearrangements and gene silencing. Activation and inactivation of *P. stutzeri* OX1 methylbenzene catabolism pathways were mediated by IS*L3*-related element IS*PsI* in response to growth substrate availability¹⁵. Transposition of IS*I411* became evident during the course of insertional activation of the promoterless *pheBA* genes in *P. putida* due to the presence of outward-directed promoters at the left end of IS*I411*⁹³. As a result of transposition, IS*I411* leaves a copy in its original location. This DNA element can produce IS circles; therefore, it was supposed that IS*I411* can follow a transposition pathway that utilises an IS circle as an intermediate.

A number of IS elements not directly associated with catabolic genes are also found in xenobiotic-degrading pseudomonads (Table 3). For example, *P. alcaligenes* NCIB 9867, isolated as dimethylphenol degrader, carries in its

Table 3. IS elements from xenobiotic-degrading *Pseudomonas* strains not linked to catabolic genes.

IS element	Family	Host (location on plasmid)	Growth	Comments substrate ^a	References
IS1383 ^b	IS110	<i>Pseudomonas</i> sp. H (pPGH1; pPGH2)	Phenol	Two copies on pPGH1, four copies on pPGH2	[116]
IS1384 ^c	IS5	<i>Pseudomonas</i> sp. H (pPGH1)	Phenol	Two copies on pPGH1 and on pPGH2; IRs are target for IS1383	[148]
IS1394	IS30	<i>P. alcaligenes</i> NCIB 9867 P25X	Xylenols	Ten copies in P25X chromosome	[264]
IS1474	IS21	<i>P. alcaligenes</i> NCIB 9867 P25X (pRA2)	Xylenols	13 copies in P25X chromosome, one on pRA2	[265]
IS1475	IS21	<i>P. alcaligenes</i> NCIB 9867 P25X	Xylenols	Targeted by IS1474	[265]
IS1491	IS21	<i>P. alcaligenes</i> NCIB 9867 P25X	Xylenols	At least 14 copies in P25X chromosome	[268]

^aCarbon source used for strain isolation.
^bBased on multiple alignments of the TnpAs, IRs and insertion sites of IS110 family elements, Lauf *et al.*¹¹⁶ defined a new family of IS elements, the IS1111 family which includes IS1383, IS1111, IS1328, and IS4321.
^cTnpA is 99% identical to TnpA1 of *P. stutzeri* AN10 found downstream of *nahD* gene¹⁷.

genome multiple copies of various IS elements that may contribute to the tremendous plasticity of its genome^{264, 265, 268}. Another example is phenol-degrading *P. putida* strain H, which carries on its plasmids six copies of IS element IS1383 and four copies of IS1384^{116, 148}. Sequence analysis of IS1383 insertions has revealed that this element has high target specificity. IS1383 integrates into the IRs of IS1384 by a site-specific recombination event which utilises a region that is formed after IS1383 circularisation¹⁴⁸. Formation of the circular intermediate upon excision of IS1383 creates a strong promoter for the transcription of the *tnpA* gene whereas the spacer between the IRs of the element contains the -10 hexamer of the promoter.

Several IS elements have been shown to form minicircle intermediates (e.g., IS911, IS21, IS3, IS1, IS2, and IS30)³⁰. It is important to point out here that although some IS elements described above (e.g., IS1411 and IS1383) probably also transpose via the formation of IS circles as intermediates, this does not mean that circle formation could be applied to all IS elements.

3.2.2. IS Elements in Plant Pathogens. IS elements described in plant pathogens are listed in Table 4. IS801, a member of the IS91 family, was originally identified in *P. syringae*¹⁹⁰. This element is widely distributed in

Table 4. IS elements in plant pathogens.

Element	IS family	Host (plasmid)	Comments	References
IS801 ^a	IS91	<i>P. syringae</i> pv. <i>phaseolicola</i> LR781 (pMMC7105)	IS element lacks terminal repeats	[190]
		<i>P. syringae</i> pv. <i>glycinea</i> PG4180 (p4180A)	Multiple copies outside coronatine (<i>cor</i>) gene clusters in p4180A	[1]
IS51	IS3	<i>P. syringae</i> pv. <i>savastanoi</i> (pIAA1)	Insertional inactivation of <i>iaaM</i> causes loss of virulence	[37]
		<i>P. syringae</i> pv. <i>glycinea</i> PG4180 (p4180A)	Flanks <i>cor</i> clusters downstream of <i>cma</i> region in p4180A	[1]
IS1240	IS3	<i>P. syringae</i> pv. <i>tomato</i> PT23 (B-plasmid)	Linked to avirulence gene D (<i>avrD</i>)	[79]
IS1240-like IS	IS3	<i>P. syringae</i> pv. <i>glycinea</i> PG4180 (p4180A)	Locates outside <i>cor</i> clusters upstream of IS870-like ISs	[1]
IS870-like IS	IS630	<i>P. syringae</i> pv. <i>glycinea</i> PG4180 (p4180A)	Flanks <i>cor</i> clusters at both sides in p4810A	[1]

^aTransposase genes nearly identical to IS801 *tnpA* have been identified in atrazine catabolic plasmid pADP-1 (four copies) from *Pseudomonas* sp. strain ADP1¹³² and in nitrobenzene-degrading *P. pseudoalcaligenes* strain JS45⁴³.

*P. syringae*⁷¹. Unlike most prokaryotic IS elements, IS801 lacks terminal repeats, a trait shared by other members of the IS91 family³⁰. IS801 does not duplicate its target. The target sequences are highly specific, having partial homology to one terminus of the element¹⁸⁷. The IS801 transposase is not a member of the DDE class transposases, and exhibits amino acid sequence homology to the transposases of *E. coli* elements IS91 and IS1294 in conserved amino acid motifs identified in the replicases of certain plasmids that replicate as rolling circles¹⁸⁷. Like other IS91 members, IS801 possibly transposes via rolling-circle transposition (see ref. [67] for a review).

3.2.3. Transposition of IS21. IS21 is the prototype of a growing family of IS elements. IS21 was originally identified in *P. aeruginosa* antibiotic resistance plasmid R68²⁵⁸. Mechanisms of transposition of this element have been extensively studied (for a review, see also refs [11, 76]). A single copy of IS21 can generate simple insertions in a pathway that is thought to involve circularisation of IS21^{185, 200}. IS21 spontaneously forms tandem repeats designated (IS21)₂. Plasmids carrying (IS21)₂ can form cointegrates with other replicons at high frequency via a cut-and-paste mechanism¹⁸⁵. The tandemly repeated copies of IS21 promote insertion of the entire plasmid in a transposition event involving the abutted terminal IRs²⁰⁰. IS21 is the first transposable element for which a transposase/cointegrase specialisation has been reported²⁰⁰. IS21 contains two genes, *istAB*, which are organised in an operon and encode transposition and helper proteins, respectively¹⁸⁵. When two IS21 elements are organised in tandem, the abutted ends form a promoter that drives the expression of the *istAB* genes in the downstream IS21 element¹⁸⁵. IstB is needed for accurate strand transfer and capture of the target DNA¹⁹⁹. Full-length IstA is transposase, sufficient to carry out insertion of single IS21 element as well as replicon fusion of (IS21)₂ plasmids. In contrast, the truncated form of transposase, a cointegrase (expressed in frame from a natural internal translation start of *istA*), essentially performs cointegrate formation but hardly any simple insertion²⁰⁰.

3.3. Tn3 Family Transposons

3.3.1. Tn3 Family Transposons in Catabolic Plasmids. Tn3 family transposons present in catabolic plasmids of different *Pseudomonas* strains are listed in Table 5. Transposition of Tn3 family catabolic transposons (e.g., Tn4653, Tn4651, and its derivative Tn4652) have been extensively studied^{86, 87, 89, 245}; (for a review, see also ref. [240]). Tn4653 and Tn4651 (Figure 2A) are nested transposons: 70-kb Tn4653 spans 56-kb-long Tn4651^{242, 243}. Tn4652²⁴² is a 17-kb derivative of Tn4651 that has a deletion of the catabolic region including *xyl* operons as a result of reciprocal recombination between two copies of

Table 5. Tn3 family transposons in catabolic plasmids.

Tn	Host (plasmid)	Catabolic substrate (genes)	Comments	References
Tn4651 ^a	<i>P. putida</i> mt-2 (pWW0)	Toluene, xylenes (<i>xyl</i> genes)	<i>impS-res-impT</i> for resolution	[243]
Tn4653 ^b	<i>P. putida</i> mt-2 (pWW0)	Toluene, xylenes (<i>xyl</i> genes)	Spans Tn4651, <i>res</i> defective, uses Tn4651 resolution system	[245]
Tn4655 ^c	<i>P. putida</i> PpG7 (NAH7)	Naphthalene (<i>nah</i> genes)	<i>impA</i> defective, site-specific integrase for cointegrate resolution	[244]
Tn4656 ^d	<i>P. putida</i> MT53 (pWW53)	Toluene, xylenes (<i>xyl</i> genes)	Mutations in left IR decrease frequency of transposition	[241]
Tn4676 ARMphe	<i>P. resinovorans</i> CAR1 (pCAR1) <i>P. fluorescens</i> (pAM10.6)	Carbazole (<i>car</i> and <i>ant</i> genes) Phenol (<i>pheBA</i>)	Transposition system similar to Tn4651 Captured laterally transferred <i>pheBA</i> , lacks transposition functions	[127] [169]
Tn5501 ^e	<i>Pseudomonas</i> sp. H (pPGH1)	None	Nearby phenol degradation genes (<i>phl</i>)	[115]
Tn5502	<i>Pseudomonas</i> sp. H (pPGH1)	None	Nearby phenol degradation genes (<i>phl</i>)	[115]

^aTransposition functions are not exchangeable with those encoded by Tn1722 subgroup transposons²⁴⁵. Deletion of catabolic region by reciprocal recombination between the two copies of IS1246¹⁸⁴ results in Tn4652²⁴². Tn4652 transposase was not exchangeable with those of the Tn1722 subgroup transposons²⁴⁵.
^bTransposition functions are exchangeable with those encoded by Tn1722 subgroup transposons²⁴⁵. Tn4654 is a derivative of Tn4653 that has deleted the catabolic region similarly to Tn4652²⁴³. Tn4654-related element has been involved in mobilisation of camphor degradation cluster in *P. putida* AC793¹²⁶ and similar element derived from alkanes degradation OCT plasmid of *P. putida* PpS5 was involved in mobilisation of non-conjugative replicons (discussed in Ref. [169]).
^cCointegration function is exchangeable with that encoded by Tn1722 subgroup transposons²⁴⁴.
^dTransposition functions are exchangeable with those encoded by Tn1722 subgroup transposons²⁴¹.
^eSequence nearly identical to Tn5501 was found in close proximity to the hydroxylaminobenzene mutase genes in *P. pseudocataligenes* strain JS45⁴³.

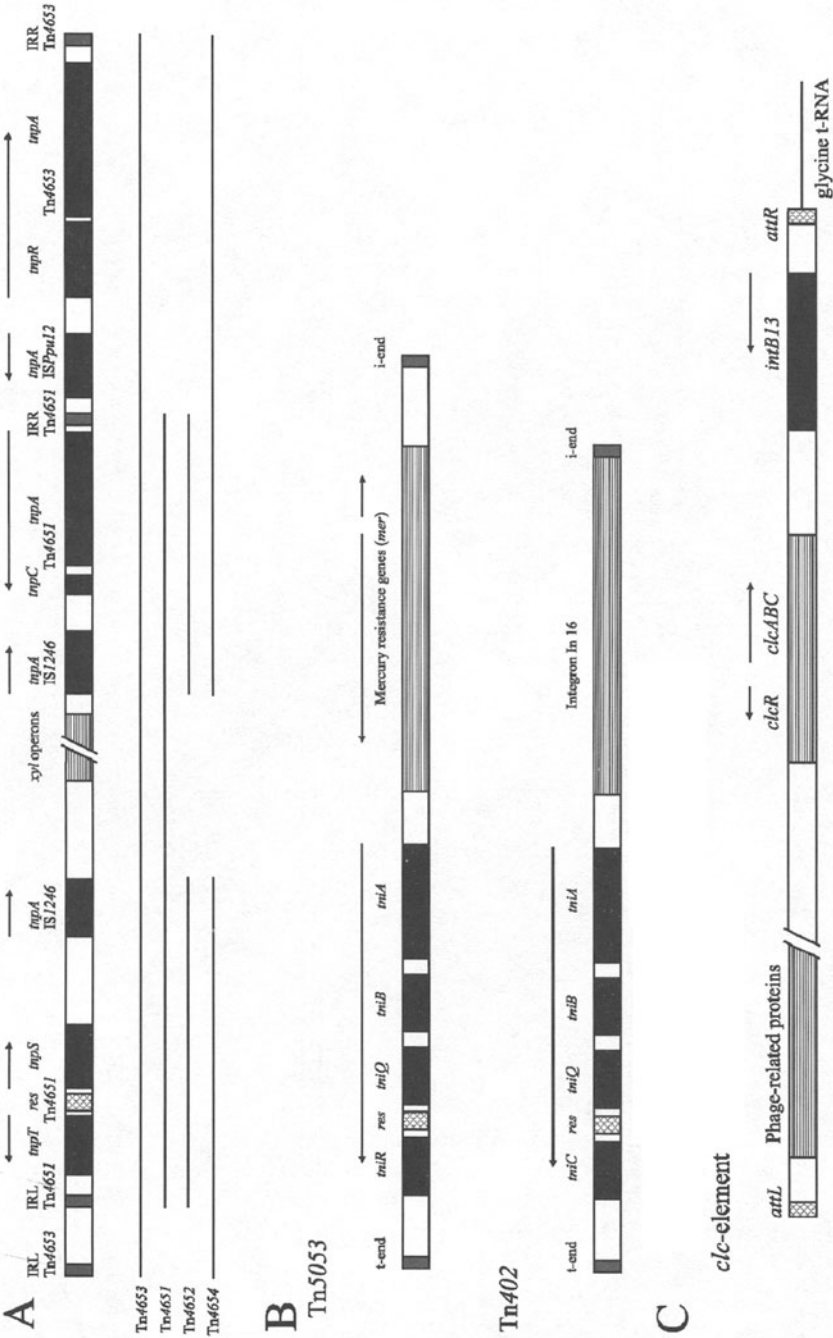


Figure 2. Genetic organisation of various transposable elements characterised in *Pseudomonas* strains. A. IS elements and transposons in TOL plasmid pWW0. Lines below the map indicate the extension of different transposons. The toluene degradation *xyI* genes (interrupted striped boxes) are located in Tn4651²⁴² that is included within another transposon, Tn4653²⁴³. IRs of transposons are marked by grey boxes and functional *res* site for Tn4651 and Tn4653 by cross-hatched box. IRs for IS elements (IS1246, IS*Ppu12*) are not shown. Genes encoding transposases (*mpR*, *mpS*) or proteins influencing activity of the formers (*mpC*, *mpT*) are shown by black boxes, and arrows indicate the direction of transcription of the genes. Note that sizes of the indicated genes and distances between these genes are not in scale. Tn4652 and Tn4654 can form as a result of reciprocal recombination between two copies of IS1246⁸⁴ resulting in one copy of IS1246 element present in these transposons. B. Structural and functional organisation of Tn5053¹⁰² and Tn402⁷⁶. The ends of transposons (t-end and i-end) are indicated by the grey box. Black boxes represent the genes encoding transposase (TniA), resolvase (TniR for Tn5053, TniC for Tn402) and auxiliary proteins (TniQ, TniB). DNA regions encoding other functions (mercury resistance operon in Tn5053 and integron In16 in Tn402 (striped boxes) are shown as well. Arrows indicate the direction of transcription of the genes. C. Schematic presentation of the *clc* genomic island⁸⁰. Integrase encoded by *intB13* near the right end of the *clc* element catalyses recombination between the 18-bp 3' end of the *gcb*-rRNA (*attB* site) and the identical 18-bp sequence *attP* originating from the junction between left (*attL*) and right (*attR*) ends of the element. Excision of the *clc* element is also mediated by IntB13 integrase, although it is not yet clear whether other auxiliary factors may be needed for that process. Expression of the integrase gene in the circular form of the *clc* genomic island is controlled by a strong constitutive promoter present in the left end of the element²⁰⁴. The striped box marks the chlorocatechol degradation gene cluster *clc* and arrow indicates the direction of transcription of the *clc* genes. The left end of the *clc* element contains an area well-conserved among various other genomic islands, and this region is supposed to be important for regulation of integrase expression (phage-related proteins, marked as striped box).

IS element IS1246¹⁸⁴. The deduced amino acid sequence of *tnpA* from Tn4652 exhibited only a moderate homology to other Tn3 family transposases except the mercury resistance transposon Tn5041¹⁰⁰, suggesting that Tn4652 and Tn5041 are distant members of the Tn3 family forming a separate subgroup⁸⁶.

A site-specific recombination system for cointegrate resolution encoded by Tn4651/Tn4652 is also unusual among Tn3-like elements. Most members of the Tn3 family transposons use for cointegrate resolution a serine recombinase that is homologous to the prototypical resolvase TnpR of Tn3 catalysing only the intramolecular recombination between the two copies of the recombination site⁷⁴. A cointegrate resolution system of Tn4652 consists of two proteins, TnpS, which contains the R-H-R-Y tetrad commonly conserved in the integrase family of site-specific tyrosine recombinases, and TnpT, which does not show any significant sequence homology to the proteins that are involved in recombination⁶⁹. The 203-bp *res* site partly overlaps the divergently transcribed *tnpS* and *tnpT* genes. Tn4652-encoded TnpS can catalyse both the intramolecular resolution and intermolecular integration reactions whereas TnpT most likely enhances the resolution frequency by interacting directly with TnpS⁶⁹.

Retrotransfer mediated by pWW0 has been demonstrated by Ramos-González *et al.*¹⁷⁸. Retrotransfer has been defined as a conjugational biparental event that leads to the capture of genetic traits from the recipient by the original host of a conjugative plasmid²²⁸. pWW0 mobilised some regions of the chromosomal DNA but not the others, suggesting that pWW0 may integrate into the genome of the recipient by a site-specific recombination¹⁷⁸. pWW0 with a mutation in the Tn4653 resolvase gene *tnpR* was unable to mobilise chromosome, which indicates that the process of retrotransfer requires Tn4653 TnpR-provided site-specific recombinase activity¹⁹².

3.3.2. Resistance Genes Associated with Tn3 Family Transposons and Integrins. Bacteria can become antibiotic-resistant either by spontaneous mutation or by acquisition of resistance determinants¹⁵⁷. Transposons and integrins encoding resistance to antibiotics are frequently carried on plasmids, but can also have a chromosomal location. Resistance transposons found in *Pseudomonas* strains are listed in Table 6.

Antibiotic resistance and heavy metal resistance determinants are frequently contained in the same plasmid⁴. Both plasmid-borne and chromosomal mercury resistance determinants often reside on transposons. A transposon originally found in enteric bacteria, the Tn3 family transposon Tn21, carries besides mercury resistance genes integron-associated genes for resistance to sulfonamide and streptomycin^{21, 75}. In *Pseudomonas* strains, certain Tn21-related transposons also carry both mercury resistance and antibiotic resistance genes⁷⁵.

Table 6. Resistance genes found in transposons of different *Pseudomonas* isolates.

Element	Host	Comments	References
Tn5393 ^a	<i>P. aeruginosa</i> MUS	Sm ^r (<i>strA-strB</i> genes)	[223]
	<i>P. syringae</i> strains ^b	Sm ^r (<i>strA-strB</i> genes)	[223]
	<i>Pseudomonas</i> sp. strains ^c	Sm ^r (<i>strA-strB</i> genes)	[223]
Tn1720	<i>Pseudomonas</i> sp. R171A	Tet ^r (<i>tetA</i> gene)	[202]
Tn1404	<i>Pseudomonas</i> sp. R9	Tet ^r (<i>tetCR</i> genes), two copies of IS26 and integron (<i>sul1</i> , <i>aadA2</i> cassettes Su ^r Sm ^r)	[202]
Tn1403	<i>P. aeruginosa</i>	Integron In28 (<i>blaP1</i> , <i>aadA2</i> , <i>cmlA1</i> cassettes, Cb ^r Sm ^r Cm ^r) in <i>res</i> site, IS6100	[167, 252]
Tn1405 ^d	<i>P. aeruginosa</i> Dalglish	Carries Cb ^r , Sm ^r , Sp ^r , Su ^r determinants	[121]
Tn501	<i>P. aeruginosa</i>	Hg ^r	[218]
Tn501v1	<i>Pseudomonas</i> sp. BS6	Hg ^r , identical to Tn501	[145]
Tn501v2	<i>Pseudomonas</i> sp. MU19-3	Hg ^r , identical to Tn501	[145]
Tn5050	<i>Pseudomonas</i> sp. LS45-3	Hg ^r , belongs to Tn21 subgroup	[145]
Tn5051	<i>P. putida</i> HU1-6	Hg ^r , belongs to Tn21 subgroup	[145]
Tn5061	<i>P. alcaligenes</i> FA8-1	Hg ^r , belongs to Tn21 subgroup	[145]
Tn5041 ^e	<i>Pseudomonas</i> sp. KHP41	Hg ^r , chimera of Tn21 subgroup and Tn4652-related elements, truncated group II intron	[100]
Tn5044	<i>Pseudomonas</i> sp.	Hg ^r	[101]
Tn5053 ^f	<i>Pseudomonas</i> sp.	Hg ^r , <i>tniABQ</i> and <i>tniR</i> for transposition; targets <i>res</i> site	[103, 144]
Tn5563	<i>P. alcaligenes</i> NCIB 9867	Encodes putative mercuric ions transport proteins	[266]
Tn402	<i>P. aeruginosa</i>	Integron In16, <i>tniABQ</i> and <i>tniR</i> for transposition; targets <i>res</i> site	[176, 144]
Tn2521	<i>P. aeruginosa</i>	Integron In33 (<i>blaP1</i> , <i>aadA1</i> cassettes, Cb ^r Sm ^r Sp ^r) in <i>res</i> site, lacks transposition genes	[166]
Tn1696	<i>P. aeruginosa</i>	Hg ^r , Su ^r , integron In4 (<i>aacC1</i> , <i>aadA2</i> , <i>cmlA1</i> cassettes, Gm ^r Sm ^r Cm ^r) in the <i>res</i> site, IS6100	[167]
Tn1412	<i>P. aeruginosa</i>	Carries integron In3n in the <i>res</i> site of Tn5563-based backbone	[167]

^aThis element was originally isolated from *Erwinia amylovora*³³.^bStrains A2, 7B12, and 8C32 carry Tn5393 in plasmids pPSR1, pPSR14, and pPSR15, respectively.^cStrains BixF6, PsR9, and PsR17 carry Tn5393 in plasmids pBIXF6, pPsR9, and pPSR17, respectively.^dTn1405 and Tn2521 are probably identical¹⁶⁶.^eTn5041-like elements have been found in many *Pseudomonas* strains¹⁰¹.^fTransposons identical or nearly identical to Tn5053 (variants v1, v3, v4 and v6 have been found in different *Pseudomonas* strains¹⁴⁵).

Transposons Tn501, Tn5053, and Tn5041 are the best-studied mercury resistance transposons in *Pseudomonas*^{22, 48, 100, 102, 144}. Mercury resistance transposon Tn5053¹⁰² transposes via replicative transposition. This transposon requires for cointegrate formation the activity of TniABQ, and TniR for the resolution of cointegrate¹⁰³. Tn5053 is closely related to Tn402 (Tn5090)¹⁷⁶. Transposons Tn5053 and Tn402 (Figure 2B) have been ascribed to a family distinct from the Tn3 family¹⁰³. Based on the binding pattern and organisation of repeats at the ends of Tn402 suggested that mechanism of transposition of Tn402 has similarity to Mu transposition⁹⁵. Most of the Tn5053/Tn402 family transposons have inserted into a common target site, a *res* region of Tn3 family elements upstream of the gene encoding a resolvase-like protein¹⁴⁴. Not all Tn3 family transposons provide target sites for Tn5053. Testing of different targets for Tn5053 transposition revealed that *res* regions of Tn1721 and Tn5044 served as good targets, while another set of diverse *res* regions, such as those of Tn1 and Tn5053 itself, did not¹⁴⁴. The *res* region served as a target for transposition of Tn5053 only in the presence of cognate resolvase expressed either *in cis* or *in trans*. Based on these findings the authors hypothesised that Tn5053 transposition may involve formation of an intermediate complex between the target DNA *res* region and the corresponding resolvase. This may also explain why the orientation of insertions of Tn5053 relative to the targeted *res* region was the same in all cases studied.

A detailed study of molecular diversity and evolutionary relationships of mercury-resistance transposons recovered from environmental bacteria has revealed that Tn21, Tn5053, and Tn5041-like transposons are widely distributed among *Pseudomonas* species all over the world^{101, 145}. Some of the Tn5041-like transposons characterised were found to carry additional genetic material resulting from distinct recombination events, such as the insertion of group II intron INT5041C, a *mer2* gene cassette or a Tn21 homologue¹⁰¹. Mosaic mercury-resistance transposons that have possibly evolved as a result of insertion of a Tn5053-like element into an ancestral Tn501-like transposon followed by deletions and other genetic rearrangements have been described in *P. stutzeri* plasmid pPB¹⁸⁶. Other chimaeras of mercury resistance transposons formed as a result of homologous or site-specific recombination have been described^{145, 270}.

Antibiotic resistance genes are frequently found in gene cassettes that can be integrated at a specific position in an integron^{182, 220}. Gene cassettes are the simplest mobile elements as they include only a single gene associated with a recombination site known as *attC*. Despite a substantial variation in length, these recombination sites have historically been referred to as 59-base elements (59-be). Among the three major classes of integrons described, the class 1 is most frequently encountered¹⁸³. A number of Tn3 family transposons in *Pseudomonas* are known to carry a class 1 integron inserted into the *res* site of the transposon (Table 6). Class 1 integrons have been found also in soil bacteria, such as *P. stutzeri*⁸⁴ and *P. putida*¹¹⁸.

3.4. Superintegrans

Ordinary integrons contain no more than three gene cassettes¹⁸². Superintegrans may contain more than 100 gene cassettes encoding diverse functions including those possibly related to pathogenesis¹³⁵. Superintegrans were recently detected also in the genomes of *Pseudomonas* species²⁴⁶. Sequence analysis of the superintegron In55044 from non-pathogenic *P. alcaligenes* strain ATCC 55044 revealed a high structural similarity to that described in *Vibrio cholerae*^{135, 246}. A large fraction of ORFs was hypothesised to encode proteins associated with cell envelope, but no antibiotic resistance cassettes were found. Similar structures were detected in other *Pseudomonas* species living in soil and aquatic habitats²⁴⁶. Repetitive sequences that were identified as 59-be-like recombination sites in *P. alcaligenes* superintegron In55044 form a family of elements that are distributed in *Pseudomonas*. The authors proposed that a single susceptible ancestral element can be possibly present in all *Pseudomonas* species, and has undergone sporadic expansion in some species while not in the others.

3.5. Conjugative Transposons and Genomic Islands in *Pseudomonas* Isolates

Catabolic operons may be transferred to a new host also as part of conjugative transposons or genomic islands (Table 7). Among these genetic elements, a 105-kb-long *clc* element (Figure 2C) of *Pseudomonas* sp. strain B13, which carries genes for chlorocatechol degradation, is one of the best-studied examples in *Pseudomonas*^{180, 248}. Transfer of the *clc* element is mediated by a P4-related integrase. The *clc* element integrates site-specifically into the chromosome of various bacterial recipients using the 3' end of glycine tRNA as an integration site. Transfer of the *clc* element to other bacteria has been observed in complex microbial communities, such as sludges from soil and wastewater treatment plants^{234, 271}. Also, when the *clc*-carrying *P. putida* strain BN210 was inoculated into a bacterial population in 3-chlorobenzoate-contaminated wastewater, the *clc* element was taken up by *P. aeruginosa* strains and by strains belonging to the genera *Ralstonia* and *Comamonas*²¹⁶.

Genomic islands are unstable DNA segments that usually carry determinants important for the survival of the bacteria in unique environmental niches. Evolution of pathogenic variants from non-pathogenic or less virulent strains is a well-documented phenomenon in many bacterial species⁷⁷. Restriction mapping of genomes of *P. aeruginosa* strains from diverse backgrounds has revealed that a significant amount of the variation among the isolates is due to insertions and deletions of large segments of genomic material^{191, 201}. Recent data have demonstrated that genomic islands play an important role in

Table 7. Conjugative transposons and gene islands in different *Pseudomonas* isolates.

Genetic element	Host	Function (genes)	Comments	References
<i>clc</i> element	<i>Pseudomonas</i> sp. B13	Chlorocatechol degradation (<i>clc</i> genes)	105 kb, conjugative transfer, site-specific integration	[180]
<i>bph-sal</i> element	<i>P. putida</i> KF715	Biphenyl and salicylate degradation (<i>bph, sal</i>)	90 kb, conjugative transfer, genetically unstable	[155]
Mobile element	<i>P. aeruginosa</i> JB2	Hydroxy- and haloaromatics degradation (<i>hyb, clc^a</i>)	Conjugative transfer,	[82]
Hrp PAI	<i>P. syringae</i> strains	Type III secretion system, important in pathogenesis	<i>clc</i> genes flanked by IS21 family elements	[2, 36]
Glycosylation island	<i>P. aeruginosa</i> PAK	Virulence factors glycosylation	50 kb, flanked by genetically diverse and unstable effector locus	[6]
PAGI-1	<i>P. aeruginosa</i> X24509	Most genes unknown, facilitates survival under stress	16 kb, glycosylates only a-type flagellin present in pathogenic isolates	[122]
PAGI-2 ^b	<i>P. aeruginosa</i>	Encodes metabolic functions and transporters	49 kb, has at least two different origins, present in many clinical isolates	[114]
PAGI-3 ^b	<i>P. aeruginosa</i>	Encodes metabolic functions and transporters	105 kb, hypervariable, integrated into tRNA ^{Gly}	[114]
Gene island	<i>P. putida</i> IsoF	AHL signal molecules (<i>lasR-rsaL-lasI</i> homologues)	103 kb, hypervariable, integrated into tRNA ^{Gly}	[219]

^a*clc* Genes are almost identical to those described in chlorocatechol degradation plasmid pAC27^{55, 62}.

^bOwens attachment sites and surrounding sequences similar to that found by analysing insertion sites of the *clc* element¹⁸⁰. PAGI-2 is present also in *Ralstonia metallodurans* CH34 chromosome¹¹⁴.

pathogenicity of *P. aeruginosa* (summarised in Table 7). Genetic material that contributes to parasitic fitness in plant pathogens^{2, 36} or enables production of *N*-acylhomoserine lactone (AHL) signal molecules²¹⁹ can also be found in genomic islands (Table 7).

3.6. Mobile Introns in *Pseudomonas*

Group I and group II introns are found in bacteria and phages, but their distribution is erratic⁵⁴. According to Edgell *et al.*⁵⁴, group I introns are more frequent in bacteria than group II introns. Group II introns are not found in phages⁵⁴. However, group II introns have been detected in a wider spectrum of phylogenetically diverse bacterial genera. Unlike eukaryotic group II introns, the bacterial group II introns described so far contain ORFs. A number of group II intron-encoded proteins contain reverse transcriptase and maturase domains but lack a recognisable endonuclease domain¹³³. Most group I introns have been discovered in cyanobacteria; only a few cases have been reported in proteobacteria⁵⁴. Recently, a novel group I intron that interrupts the anticodon loop of the tRNA^{Leu}(UAA) gene was described in genus *Pseudomonas* bacteria living in deep surface environments²⁵¹.

Group II introns have been isolated in different *Pseudomonas* species. Introns discovered in *P. alcaligenes* NCIB 9867, *P. putida* NCIB 9869, and *P. putida* KT2440 are closely related^{267, 269}. Although the *Pseudomonas* introns lacked an endonuclease domain, the group II introns found in *P. alcaligenes*, Xln6 and Xln3, were shown to have transposed from the chromosome to the plasmid^{267, 269}. The fact that a group II intron can retrotranspose to new sites in a RecA-dependent fashion, in the absence of endonuclease activity, was experimentally demonstrated in the case of group II intron from *Lactobacillus lactis*⁴⁰. A homologue of the *P. alcaligenes* group II intron Xln6 found in *P. putida* KT2440 (95% identity to the Xln6 sequence) was identified downstream of the *rpoS* gene in *P. putida*²⁶⁹. Eight almost identical copies of Xln6 homologue are present in the chromosome of KT2440 (Table 1). This indicates that after the transfer of Xln6 homologue to *P. putida*, this element was active and was possibly transposed into many sites.

3.7. Retrons in *Pseudomonas*

Retrons are found in a wide variety of Gram-negative bacteria and are responsible for the production of an unusual satellite DNA called msDNA²⁶². A reference in the GenBank database⁵¹ indicates that an ORF found in *P. syringae* pv. *tomato* str. DC3000 may be a part of msDNA-producing retron.

4. INVOLVEMENT OF TRANSPOSABLE ELEMENTS IN MUTAGENESIS

Transposable elements contribute significantly to spontaneous mutagenesis in bacteria: insertional activation of adjacent genes, inactivation of genes by interruption, and rearrangement of the host genome are well documented^{30, 66, 128}. While transposition can cause a diverse set of mutations (most of them are potentially deleterious), a few favourable mutations may also occur, leading to adaptation of populations to a new environment. The following gives an overview of transposable elements-induced genetic changes that are involved in the genetic adaptation of *Pseudomonas* (summarised in Table 8).

The positive effect of gene interruption by an IS element for the survival of bacteria under harsh environmental conditions was established in the case of transposition of an IS21-family element ISS12 in *P. putida* strain S12²⁵⁶. This IS element was shown to play a key role in the tolerance of *P. putida* S12 to sudden toluene stress by inactivating the transcriptional repressor of the solvent efflux pump genes *srpABC*.

P. putida PP3 has evolved the ability to utilise halogenated alkanolic acids such as 2-mono- and 2,2-dichloropropionic acids (2MCPA, and 2,2DCPA, respectively) during chemostat selection²⁰³. This event led to the expression of two dehalogenases DehI and DehII, and associated permeases. The evolved *P. putida* strain PP3 became sensitive to certain toxic, non-metabolisable substrates like dichloroacetic acid (DCA). Exposure of the strain PP3 to DCA led to the accumulation of DCA-resistant (and 2MCPA negative) mutants caused by the movement of the gene for DehI within the transposon designated *DEH*^{211, 235–237}. The ability to utilise 2MCPA was readily restored under changed environmental conditions (in the presence of growth-supporting substrate 2MCPA)²³⁵. As already discussed above, recent data have revealed that the *DEH* element is a composite transposon on which *dehI* and its cognate regulatory gene *dehR_I* are flanked by copies of *ISPpu12*²⁵⁴. *ISPpu12* is also present in TOL plasmid pWW0²⁶⁰ and its transposition is probably associated with many toluene catabolic mutants obtained during earlier studies on pWW0.

Activation and inactivation of *P. stutzeri* OX1 methylbenzene catabolism pathways were also mediated by transposition of IS elements¹⁵. *P. stutzeri* strain OX1 was able to grow on toluene and *o*-xylene as the sole carbon and energy source but *m*-xylene and *p*-xylene were not used for growth by this strain. Nevertheless, spontaneous mutants appeared that had acquired the ability to grow on two latter substrates but had lost the ability to utilise the *ortho* isomer. Revertants that could utilise all the three isomers of xylene were isolated as well. The metabolic versatility in OX1 and its derivatives was brought about by genome rearrangements mediated by *ISPsI*: This DNA element can transpose into and precisely excise out of catabolic genes, causing inactivation or activation of respective catabolic functions¹⁵. The authors

Table 8. Genetic adaptation of *Pseudomonas* under environmental stress.

Organism	Mutation	References
<i>I Involvement of mobile elements</i>		
<i>P. aeruginosa</i>	IS6100-induced insertion-inversion mechanism has disrupted the genes <i>wbpM</i> , <i>pilB</i> or <i>mutS</i> in isolates found in the lungs of chronic cystic fibrosis (CF) patients.	[110]
<i>P. putida</i> PaW85	Activation of the promoterless <i>pheBA</i> genes by promoter creation during transposition of Tn4652 from PaW85 chromosome into a plasmid carrying the <i>pheBA</i> genes.	[89, 96]
<i>P. putida</i> PaW85	Activation of the promoterless <i>pheBA</i> genes by intramolecular transposition of IS1411 carrying outward-directed promoters on its left end.	[93, 198]
<i>P. putida</i> PP3	Switching on and off the dehalogenase system by <i>DEH</i> element transposition.	[211, 235]
<i>P. putida</i> S12	Inactivation of transcriptional repressor of the solvent efflux pump genes <i>srpABC</i> by <i>ISS12</i> .	[256]
<i>P. stutzeri</i> OX1	Switching on and off methylbenzene catabolism pathways by transposition of <i>ISPs1</i> .	[15]
<i>II Other mutagenic mechanisms</i>		
<i>P. aeruginosa</i> PAO1	Constitutive expression of antibiotic efflux pump MexAB-OprM in multidrug-resistant mutants selected in the presence of tetracycline.	[3]
<i>P. aeruginosa</i> 57RP	Mutants adapted to biofilm formation, highly reversible.	[46]
<i>P. aeruginosa</i> PA14	Antibiotic-resistant phenotypic variants with enhanced ability to form biofilm in the lungs of CF patients.	[50]
<i>P. aeruginosa</i>	Porin gene <i>oprD</i> inactivation associated with carbapenem resistance in clinical and environmental isolates.	[170]
<i>P. fluorescens</i> SBW25	Rapid diversion of a population in a spatially heterogeneous environment.	[177]
<i>P. putida</i> PaW85	Activation of the promoterless <i>pheBA</i> genes by promoter creation as a result of point mutations	[96, 198]
<i>P. putida</i>	Appearance of mutants able to use novel growth substrates was stimulated by UV response genes of CAM-OCT plasmid.	[138]
<i>P. tolaasii</i>	Switching between the pathogenic smooth and non-pathogenic rough forms due to the reversible duplication of a 661-bp element within the <i>pheN</i> locus.	[210]
<i>Pseudomonas</i> sp. CF600	Mutations in <i>dmpR</i> encoding regulator of methyl phenol degradative pathway enhanced the ability of DmpR to respond to 4-methylphenol.	[197]

supposed that spontaneous switching one of the two alternative catabolic pathways (*o*-xylene catabolism vs *m*- or *p*-xylene catabolism) either on or off enables to avoid accumulation of toxic metabolites in mutant cells if a mixture of three xylene isomers is present in the medium.

Many IS elements and transposons carry outward-directed promoters or promoter modules (e.g., the -35 hexamer)^{66, 128}. Transposition of such transposable elements upstream of silent genes can activate transcription of these genes. The Tn3 family transposon, Tn4652, is a 17-kb derivative of the 56-kb toluene degradation transposon Tn4651²⁴². Tn4652 is found in the chromosome of *P. putida* KT2440 and in its isogenic strain PaW85, and can activate transcription of silent genes by creating fusion promoters^{89, 96, 158}. The fusion promoters are created as junctions between the -35 hexamer provided by the terminal inverted repeats of Tn4652 and the -10 hexamers in the target DNA for the transcription of promoterless phenol degradation genes in *P. putida* strain PaW85. Analysis of the sequence of different fusion promoters has indicated that the DNA synthesis that occurred on transposition of Tn4652 might be mutagenic. Three insertions out of six different Tn4652 transposition sites studied contained point mutations in the copy of the direct repeat, which served as a sequence for the -10 hexamer for the fusion promoter¹⁵⁸. However, the point mutations identified were most likely under positive selection in starving population of bacteria selected for growth on phenol because they made the sequences of potential -10 hexamers more similar to the consensus sequence of sigma⁷⁰-recognised promoters. Another mobile element, IS1411, is also able to activate the promoterless phenol degradation genes *pheBA* by inserting upstream of these genes⁹³. Activation of transcription of the *pheBA* genes occurred from outwardly-directed promoters present on the left end of IS1411⁹³.

Variations in the copy number of a mobile element can influence gene expression. For example, the *clc* element is able to undergo amplification by producing multiple tandemly arranged copies¹⁷⁹. Transfer of the *clc* element into toluene-metabolizing *P. putida* strain F1 created a hybrid pathway allowing the transconjugants to utilise chlorobenzene as a growth substrate. Amplification of the *clc* element was a prerequisite for growth on chlorobenzene. The mechanism of tandem amplifications in F1 transconjugants is still unclear. The amplified structures in transconjugants were quite unstable and were deamplified under non-selective conditions by recombinational deletions between tandemly arranged copies. If deamplification had finally ended in two nonadjacent copies, no new amplification cycles occurred under selection for growth on chlorobenzene¹⁷⁹.

Chromosomal rearrangements such as large inversions of DNA segments, caused in many cases by mobile DNA elements, may also be involved in adaptation of bacteria to a new environment. Such rearrangements have been frequently described among human pathogens⁸⁸. For example, studies by Kresse *et al.*¹¹⁰ have revealed that IS6100-induced large chromosomal inversions (LCIs) represent one of the mechanisms that can lead to the adaptation process of *P. aeruginosa* to cystic fibrosis (CF) lung habitat. LCIs have played

a role in genome variability and adaptation to novel ecological niches also in other pseudomonads, for example, in *P. stutzeri*⁷⁰.

Mobile introns present in bacterial genomes may serve as potential sources of change in genetic material as well. The involvement of group II introns in generation of catabolic mutants has been discussed by Yeo *et al.*²⁶⁷. *P. alcaligenes* catabolic mutants unable to degrade 2,5-xyleneol contained genetic rearrangements possibly due to the presence of two copies of group II intron Xln6 surrounding 2,5-xyleneol catabolic genes. Analysis of genetic rearrangements in the mutants indicated that these catabolic genes could be deleted as a result of recombination between the copies of Xln6. Disruption of genes for gentisate pathway by the intron Xln6 was also suggested in ref. [267].

5. REGULATION OF TRANSPOSITION IN *PSEUDOMONAS*

To avoid potentially deleterious effect of transposition to the host genome, the frequency of transposition in a cell is down-regulated, both by transposon-encoded and host-encoded factors. Transposition may be regulated by controlling transposase expression (transcriptional, translational, and/or post-translational control mechanisms) and also by factors that affect the transposition process itself (for a recent review, see ref. [30]). Under stressful environmental conditions, however, the increase in genetic diversity caused by a movement of transposable elements is one of the mechanisms allowing genetic adaptation of bacteria to a new environment²⁰⁵.

Increased transposition frequency may be the result of occasional inactivation of transposition control mechanisms but it can also be a regulated process. So far, there are only few published studies about regulation of transposition under stressful conditions. Study of the regulation of transposition of Tn3 family transposon Tn4652 originated from TOL plasmid pWW0 is one of the best-studied examples in *Pseudomonas*. Transposition of Tn4652 seems to be restricted to *Pseudomonas* hosts because of a requirement of a *Pseudomonas*-specific host factor(s) for the transcriptional activation of the *tnpA* gene promoter⁸⁶. The fact that some other groups have demonstrated transposition of Tn4652 in *E. coli*^{104, 242, 245} could be explained by the presence of putative promoter sequences upstream of the natural promoter of *tnpA* in constructs used in the transposition assay. The amount of Tn4652 TnpA is down-regulated by the Tn4652-encoded TnpC⁸⁷. Transposition of Tn4652 is an exclusively stationary-phase-specific event and the maximum rate of transposition frequency of this element is achieved after few days of starvation of cells^{89, 96}. Transposition of Tn4652 is strongly suppressed in *P. putida* defective for stationary phase-specific sigma factor RpoS: RpoS is required for

transcription initiation from the Tn4652 *tnpA* promoter⁸⁹. Binding of IHF upstream to the *tnpA* promoter has also positive effect on the expression of the *tnpA* gene⁸⁶. IHF binds both ends of Tn4652^{86, 233}. DNase I footprinting studies have revealed that binding of IHF to the ends of Tn4652 is necessary for transposase binding at the termini of Tn4652. Transposition of Tn4652 is drastically decreased in *P. putida* IHF-defective strain (H. Ilves, R. Hõrak, R. Teras, and M. Kivisaar, unpublished results). Summing up, these results support the idea that at least in the case of some transposable elements, activation of transposition under stressful conditions can be an inducible process, not merely the malfunction of host defence mechanisms.

There are also other reports on studies of transposition in *Pseudomonas* demonstrating that transposition may be stress-induced. For example, it seems that some late-starvation signal can be needed for the activation of IS1411¹⁹⁸. However, converse to Tn4652, the role of RpoS in regulation of transposition of IS1411 was opposite: Transposition frequency of IS1411 increased drastically in *P. putida* PaW85 RpoS-deficient mutant. Starvation-induced transposition has been also detected in the case of movement of *DEH* elements in *P. putida* strain PP3^{211, 235}. Certain environmental conditions such as changes in temperature and pH affected the transposition frequency of these elements as well⁸⁵. Unfortunately, studies of the molecular mechanisms of regulation of transposition of *DEH* elements are so far missing. The phenomenon observed by Slater *et al.*²¹¹ showing that trichloroacetic acid, which is neither the growth substrate nor the growth-inhibitor for cells of PP3, also drastically stimulates the transposition, would certainly deserve further investigations to elucidate the link between environmental signals sensed by bacterial cells and the frequency of transposition.

6. POINT MUTATIONS IN BACTERIA

Mutations not associated with the movement of transposable elements originate from various sources, including errors made during replication of undamaged template DNA, mutagenic nucleotide substrates, and endogenous DNA lesions¹²⁹. Replication fidelity reflects the combined actions of DNA polymerase, proofreading exonuclease, and post-replication error-correcting DNA repair pathways. The integrity of DNA is continually threatened by ubiquitous agents such as heat, irradiation, reactive oxygen species and chemicals that break the DNA backbone or alter the chemical structure of DNA bases. Cells exposed to a variety of DNA-damaging agents result in a dramatic increase in the mutation rate⁶⁴. DNA polymerase III may stall when attempting to copy a lesion. Continued unwinding of the DNA ahead of the blocked replication fork could provide a region of single-stranded DNA, allowing

assembly of an activated RecA nucleoprotein filament capable of inducing the SOS response. *E. coli* has five DNA polymerases. Three of these polymerases, pol II, pol IV, and pol V are induced as a part of SOS regulon in response to DNA damage⁷². These DNA polymerases can continue DNA replication when replication fork is collapsed at a blocking lesion, but the DNA synthesis by pol IV and pol V is error-prone^{72, 227}.

All organisms have multiple overlapping DNA repair pathways⁵⁵. The three major DNA repair pathways are base excision repair (BER), nucleotide excision repair (NER), and mismatch repair (MMR). Several additional pathways, including direct damage reversal, transcription-coupled repair, recombinational repair, and double-strand break repair also play important roles in preserving DNA integrity¹⁹⁵.

6.1. RecA Functions in *Pseudomonas*

RecA protein is a multifunctional protein that is essential to three distinct but related biological processes such as general genetic recombination, regulation of the coordinated expression of many unlinked genes in response to DNA damage (SOS response), and the error-prone replicative bypass of DNA lesions (reviewed in refs [64], [72], [226], Figure 3). In *E. coli* SOS response, the expression of more than 30 proteins involved in DNA replication, repair, and control of cell division is induced after cells are exposed to DNA damaging agents^{57, 64}.

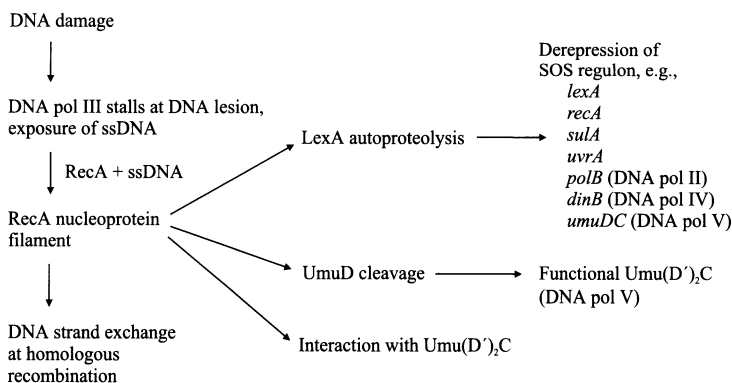


Figure 3. RecA of *E. coli* is involved in homologous recombination, SOS induction, and error-prone replicative bypass of DNA lesions⁶⁴. To function in these processes, RecA must assemble on ssDNA to form a nucleoprotein filament. The coprotease activity of RecA is necessary for catalysis of self-cleavage of LexA repressor and UmuD. UmuD forms a complex with UmuC. Only (UmuD')₂C complex resulting from cleavage of UmuD to UmuD' functions as DNA polymerase (pol V). RecA nucleoprotein filament also targets the (UmuD')₂C complex at the site of a DNA lesion, thereby facilitating translesion DNA synthesis by (UmuD')₂C.

The *P. aeruginosa* *recA* gene was shown to be capable of complementing *recA* mutations in *E. coli*¹⁰⁹. Gene products with activities similar to that of the RecA protein of *E. coli* have been identified also in other *Pseudomonas* species, such as *P. putida*¹²⁴, *P. fluorescens*⁴⁴, *P. cepacia*¹⁵¹ and *P. syringae*⁸³. Among these proteins, the RecA protein from *P. aeruginosa* has been most extensively studied. A distinct characteristic of the RecA protein from *P. aeruginosa* is its hyper-recombinogenic activity in *E. coli*. The *P. aeruginosa* RecA, when introduced into *E. coli* RecA-deficient mutant, increased the frequency of recombination exchanges about six times¹⁵². Subsequent studies⁸ suggested that the intensity of subunit-subunit interactions in the RecA filament structure can be one of the factors influencing the frequency of recombination exchanges by the RecA protein.

The RecA/ssDNA nucleoprotein filament functions as a coprotease that stimulates LexA autoproteolysis¹²³. The resulting decrease in the cellular pool of LexA results in the induction of SOS regulon. LexA repressor binds the operators (called SOS boxes) of different SOS genes, which are also termed *din* (damage-inducible) genes⁹⁹. The results presented in ref. [68] suggested that the *lexA* gene and protein are conserved in enterobacteria and pseudomonads. Conservation appeared also in the case of SOS boxes present upstream of the *lexA* genes compared in ref. [68]. The timing, duration, and level of expression can vary for each LexA-regulated gene, depending upon the location and binding affinity of the SOS boxes relative to the strength of the promoter. Therefore, some genes may be partially induced in response to even mild levels of DNA damage (e.g., of endogenous origin), while the other genes appear to be induced only if a high or persistent DNA damage is present in the cell (ref. [39] and references therein). Although there are some published reports on analysis of expression of DNA damage-induced promoters in *Pseudomonas*^{27, 253}, systematic studies have not been done.

6.2. Error-Prone DNA Polymerases in *Pseudomonas*

As mentioned above, the error-prone DNA polymerases pol IV (*dinB*(*P*) gene product) and pol V (encoded by *umuDC* genes) are induced as part of the SOS response. The *dinB*-encoded DNA polymerase IV (pol IV) belongs to the recently identified Y-family of DNA polymerases¹⁶². Like other members of this family, pol IV is involved in a translesion synthesis and mutagenesis⁶³. DNA sequences homologous to *dinB* were found in the genome of a wide range of bacteria including representatives of *Pseudomonas* species¹⁶². Analysis of complete genome sequences of *P. putida* KT2440 and *P. aeruginosa* PAO1 indicates that putative pol IV homologues in these strains are closely related (77% identity), and have 50% identity with *E. coli* pol IV sequence.

Exposure of *E. coli* to UV or DNA-damaging chemicals causes a 100-fold increase in the mutation frequency. In the cells lacking functional pol V, UV-induced mutations occur only at a low frequency; these cells are also more sensitive to UV radiation than the wild type cells^{212, 226}. The pol V-catalysed error-prone translesion synthesis takes place when all error-free processes to overcome replication block at a DNA lesion site have failed^{226, 232}. It has also been argued that induced mutagenesis could help cells to survive periods of extreme environmental stress by acting as a mechanism of genetic adaptation of microbial populations¹⁷⁴. Within Gram-negative bacteria, the genus *Pseudomonas* species examined so far lack chromosomally encoded pol V (for comparison of enterobacteria and pseudomonads, see also Table 9). Yet, many natural isolates of *Pseudomonas* strains contain plasmids that mediate UV-mutagenesis^{120, 138, 225}.

Sundin *et al.*²²⁵ have isolated and characterised the plasmid-encoded *rulAB* genes in *P. syringae* that confer UV tolerance; these genes are supposed to play an important role in protecting bacteria in the phyllosphere from the DNA-damaging effects of UV-B radiation²²⁴. The *rulAB* genes were able to complement the *E. coli umuDC* mutant restoring the UV mutagenesis phenotype, which indicates that these genes may be functionally similar to the *E. coli umuDC*¹⁰⁵.

Some antibiotic resistance plasmids, for example, R2, pPL1, R931, and pMG15 in *P. aeruginosa* strains were also shown to carry genes for UV mutagenesis^{119, 120, 208}. Bacterial DNA is also a target for the stress of freezing and thawing, which introduces breaks in DNA strands⁵. Freezing and thawing has been shown to be mutagenic to a bacterium²⁶. It is interesting to note that the studies on *P. aeruginosa* plasmid pPL1 have indicated that the genes that are responsible for protection against UV light can also protect the cells against X-ray and freeze-thaw damage²⁵⁹.

Genes conferring UV tolerance and UV-induced mutability have been localised on large catabolic plasmids in *P. putida*¹³⁶. A 300-kb IncP2 plasmid CAM-OCT from *P. putida*, which carried genes for *n*-alkanes and camphor utilisation, enhanced both survival and mutagenesis after UV irradiation of *P. putida* and *P. aeruginosa* cells¹³⁶. Interestingly, the UV response genes encoded by the CAM-OCT plasmid seem to be regulated differently from those present on other plasmids: The CAM-OCT plasmid enhanced both survival and mutagenesis in *P. aeruginosa* after UV irradiation by a RecA-independent mechanism¹³⁷. Drawing parallels with other published data, there are several proteins induced by DNA damage in bacteria that are not directly regulated by RecA or LexA¹⁰⁸. The mechanism of activation of respective genes on CAM-OCT plasmid, however, is still unknown. McBeth and Hauer¹³⁸ have shown that the UV response genes cloned from CAM-OCT plasmid increased the frequencies of *P. putida* mutants capable of using new carbon sources such

Table 9. Error-prone DNA synthesis and major DNA repair systems in enterobacteria and pseudomonads.

	Enterobacteria ^a	Pseudomonads ^b
Error-prone DNA synthesis	Genes for error-prone DNA polymerases pol IV (<i>dinB</i>) and pol V (<i>umuDC</i>) located in the chromosome ^c and are SOS-induced ⁷² . The <i>umuDC</i> homologues have been identified in plasmids as well, and they may be differently regulated (see, e.g., ref. [111]).	DNA pol IV is chromosomally encoded, <i>umuDC</i> homologues have been identified only in plasmids. ^{105, 162, 225} . Mechanisms of regulation are unknown.
GO repair system	MutT, MutM, and MutY work in concert to overcome the effects of oxidative damage of guanine (GO) ¹⁴¹ .	Functional homologues to <i>E. coli</i> MutT, MutM, and MutY have been reported ^{165, 198} .
MMR	MutH endonucleolytic activity is activated by a complex of MutS, MutL, and mismatched DNA ^{80, 131} . UvrD helicase unwinds the duplex DNA molecule at the nick.	MutH-encoding homologue is lacking. <i>P. aeruginosa</i> <i>mutS</i> , <i>mutL</i> , and <i>uvrD</i> are unable to complement <i>E. coli</i> MMR-deficient mutants ¹⁶³ .
NER	UvrA ₂ B complex scans DNA damage, UvrB and UvrC are endonucleases that cut DNA on different sides of the lesion ¹⁹⁶ . UvrD helicase unwinds the damaged oligonucleotide.	Homologues to <i>E. coli</i> genes encoding NER have been reported, but some of the <i>uvr</i> genes may be differently regulated ¹⁸⁸ . Activities of UvrD in NER and MMR might be different ¹⁶³ .

^aResults obtained from studies of *E. coli* and *Salmonella typhimurium* are summarised.

^bResults obtained from studies of *P. aeruginosa*, *P. putida*, and *P. syringae* are summarised.

^c*E. coli* that has been used for the study of role of pol IV in mutability, carries two copies of *dinB*, one in the chromosome and one in the F' plasmid¹³⁹.

as ethyl lactate or 3-methyl-3-buten-1-ol approximately by one order of magnitude. Hence, the presence of genes responsible for UV mutagenesis on a catabolic plasmid might give a bacterial cell a potential to expand its growth substrate range also in natural environments. A large catabolic plasmid TOL plasmid pWW0 in *P. putida* also confers UV-induced mutability. The genes conferring UV mutability are able to complement *E. coli* pol V-deficient mutant (M. Tark, K. Tarassova, G. Kivi, R. Tegova, A. Tover, and M. Kivisaar, unpublished results).

6.3. DNA Repair Pathways in *Pseudomonas*

Reactive oxygen intermediates are produced during aerobic respiration and other metabolic processes. Also, pathogenic bacteria are exposed to exogenous reactive oxygen intermediates during infection of the host¹⁴³. Defence against oxidative stress is provided by multiple mechanisms including detoxification of reactive oxygen species and repair of oxidatively damaged DNA. *P. aeruginosa* is the first micro-organism for which a genetic link between an oxidative stress gene and a DNA repair gene has been observed¹⁶⁰. A *P. aeruginosa* *oxyR* homologue, which encodes one of the key regulators modulating oxidative stress in bacteria, was found in an operon with *recG*, encoding a homologue of the *E. coli* RecG helicase, which is an ATP-dependent DNA recombinase implicated in DNA replication, recombination, and repair⁹⁴. The *P. aeruginosa* RecG-deficient mutant was hypersensitive to oxidative stress and UV irradiation¹⁶⁰.

Bridges *et al.*²⁰ have proposed that oxidized guanine residues, including 7,8-dihydro-8-oxoguanine (GO), constitute an important source of spontaneous mutation. To protect their genomes from oxidative DNA damage, bacterial cells have evolved efficient repair systems including DNA glycosylases MutY, MutM (Fpg), and MutT protein which hydrolyses GO¹⁴¹ (Figure 4). The function of MutM glycosylase is to remove mutagenic GO from the damaged DNA, and MutY glycosylase provides defence by removing A misincorporated opposite GO or G following DNA replication¹⁴¹. Cells that lack activity of these glycosylases have elevated rates of G:C to T:A transversions¹⁵⁴. The GO repair system has mainly been studied in *E. coli*, but the results of some recently published papers indicate that the GO repair system is an important defence system against the oxidatively damaged DNA also in *Pseudomonas* species^{165, 198}. The *mutT*, *mutM*, and *mutY* genes of the GO repair system of *P. aeruginosa* PAO1 were able to complement the corresponding *E. coli* GO repair-deficient strains¹⁶⁵ (Table 9). The deduced amino acid sequence of *P. putida* *mutY* homologue exhibited 78% identity with MutY sequence of *P. aeruginosa*, and the frequency of spontaneous Rif^r mutants increased approximately 60-fold in the MutY-defective strain PaW85 compared to the

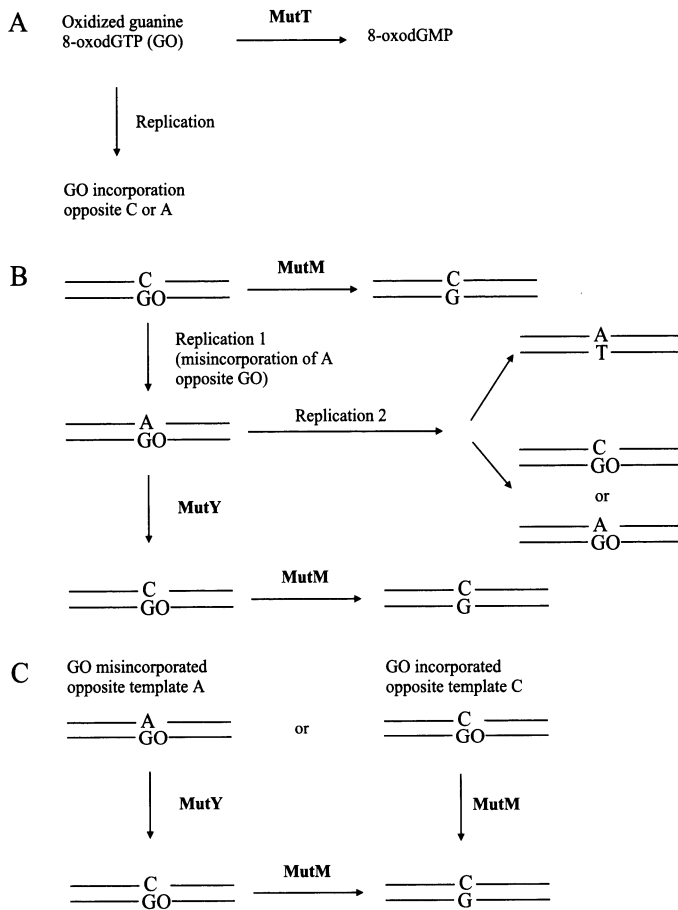


Figure 4. GO repair system in bacteria¹⁴¹. Several enzyme activities work in concert to overcome the effects of oxidative damage of guanine (GO). **A.** Oxidised guanine 8-oxodGTP is removed from the deoxynucleotide pool by MutT, which hydrolyses 8-oxodGTP to 8-oxodGMP. **B.** When a GO lesion has occurred in DNA, DNA synthesis past GO can result in the misincorporation of adenine opposite the damaged guanine²⁰⁷. MutM glycosylase removes mutagenic 8-oxoG from the damaged DNA. The glycosylase has an associated AP (apurinic) endonuclease activity that cuts the phosphodiester backbone. Exonuclease processing of the gap generated by MutM creates a substrate that can be repaired by DNA polymerase I and ligase. If MutM does not remove 8-oxoG from DNA, DNA synthesis past GO (replication 1) may result in the misincorporation of adenine opposite GO. An adenine DNA glycosylase MutY (also with associated AP endonuclease activity) removes the misincorporated adenine from A:GO mispair. Repair synthesis then results in the formation of a structure with C paired with GO, which is the substrate for MutM. If MutY does not remove A that has mistakenly paired with 8-oxoG, the second round of DNA replication (replication 2) will fix the G:C to T:A transversion (DNA polymerase inserts correct T opposite A). **C.** If replication occurs with 8-oxodGTP in the deoxynucleotide pool, inaccurate replication can result in either the misincorporation of this nucleotide opposite template adenine or the incorporation of it opposite template cytosine¹³⁰. The resulting A:GO and C:GO mispairs would be substrates for MutY and MutM glycosylases. If MutY removes A from A:GO mispair, this pathway will result in base substitution.

wild-type *P. putida*¹⁹⁸. The activity of MutY was shown to be important also in avoidance of mutations accumulating in starving cells of *P. putida*¹⁹⁸.

Methyl-directed mismatch repair (MMR) pathway corrects base–base and insertion/deletion mismatches that have escaped the proofreading function of replicative DNA polymerase or are produced by error-prone DNA polymerases^{80, 131} (Figure 5). The MutS protein recognises and binds to mismatches²²². In *E. coli*, together with MutL and in the presence of ATP, the MutS activates a third MMR protein, the MutH endonuclease^{7, 73}. MutH cleaves the transiently unmethylated daughter strand at hemimethylated GATC sequences. Various exonucleases and the UvrD helicase complete the excision of the target strand. The excision step leaves long gaps, up to several thousand bases, which are repaired by DNA polymerase pol III and DNA ligase¹³¹. Although the genes encoding MutS and MutL homologues have been found in a wide diversity of bacteria, only close relatives of *E. coli* encode MutH homologues⁵⁵. Based on the relationship between MutH and the restriction enzyme *Sau3AI* and the structural similarity to the other type II restriction enzymes⁹ it is reasonable to suppose that MutH analogues may have evolved from different restriction systems. *Pseudomonas* species also lack the *mutH* homologue. The other genes for MMR pathway (*mutS*, *mutL*, and *uvrD*) have been recently isolated and characterised in *P. aeruginosa*¹⁶⁴. Interestingly, despite a high homology to the corresponding *E. coli* sequences, none of the MMR genes of *P. aeruginosa* PAO1 complemented the increased mutation frequencies of *E. coli* strains defective for *mutS*, *mutL*, or *uvrD*¹⁶³ (Table 9). This suggests that complex interactions required between MMR enzymes and other proteins may be species-specific. The *mutS* gene of *P. putida* characterised in¹¹² was unusual due to the lack of the sequence encoding approximately 300 amino acids from the N-terminal part of MutS. However, analysis of complete genome sequence of *P. putida* strain KT2440 demonstrates that this strain has a full-length *mutS* gene.

Nucleotide excision repair (NER) removes a wide variety of lesions from bacterial genomes through a set of coordinated reactions carried out by multiprotein complex, the UvrABC endonuclease¹⁹⁶ (Figure 6). The UvrD helicase (which participates also in MMR pathway), in concert with DNA polymerase I, removes the damaged 12–13-nt oligonucleotide. Then a repair patch is synthesised by pol I, and sealed by DNA ligase. Characterisation of *uvrD* mutants of *P. aeruginosa* have revealed three amino acid substitutions within the conserved ATP binding site of the deduced UvrD polypeptide that abolished MMR activity but did not affect UvrABC-mediated excision repair¹⁶³. This is the first published report indicating that the activities of UvrD in NER and MMR might be different (Table 9).

The expression of certain DNA repair systems may be differently controlled in *E. coli* and in *Pseudomonas* (Table 9). For example, in *E. coli* the *uvrA* and *uvrB* genes are members of the SOS regulon and are induced by DNA damage, whereas the *uvrC* is constitutive⁶⁴. In contrast, the *uvrB* homologue of

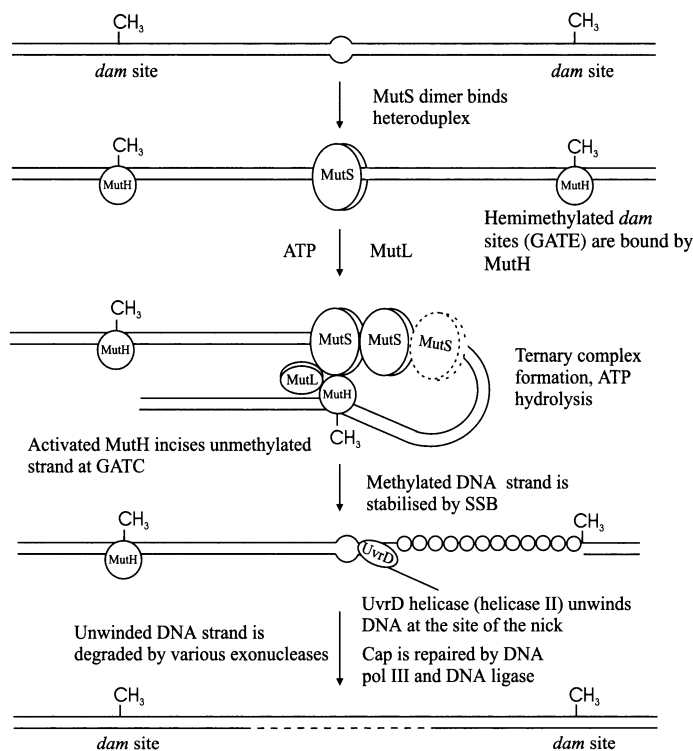


Figure 5. MMR pathway in *E. coli*^{80, 131}. The first step is the detection of a mismatch through the binding of MutS dimer to heteroduplex DNA. Only one of the two subunits contacts the mispair. The strand discrimination signal in the *E. coli* MMR system is provided by the transiently unmethylated state of newly synthesised DNA. MutH protein cleaves the unmethylated strand of a hemimethylated *dam* methylation site, thereby marking the nicked strand for exonucleolytic removal and resynthesis. MutH endonucleolytic activity is activated by a complex of MutS, MutL, and mismatched DNA, and requires ATP hydrolysis. MutH can nick DNA on either side of a mismatch. The activity of UvrD helicase (DNA helicase II) is needed to unwind the duplex DNA molecule. MutL also directly interacts with UvrD (this interaction is not shown in the figure). MutL is speculated to load the UvrD helicase onto the nick in a directional manner so that DNA unwinding proceeds toward the mismatch. The single-stranded template strand is protected by a single-strand binding protein SSB. Resynthesis is performed by DNA polymerase III and repair is completed by ligation of the remaining nick by ligase. Both the translocation model and the sliding clamp model (see, e.g., ref. [131] for a review) assume that MutS is released from the mismatch. Alternatively, results in ref. [91] suggest that MutS loading onto a mismatch induces the formation of a higher order complex containing multiple MutS homodimers, which can productively interact with MutL in ATP-hydrolysing conditions and generate a specific ternary complex, which might communicate with MutH. The growth of a MutS–DNA complex at the mismatch is illustrated only on one side of the mismatch.

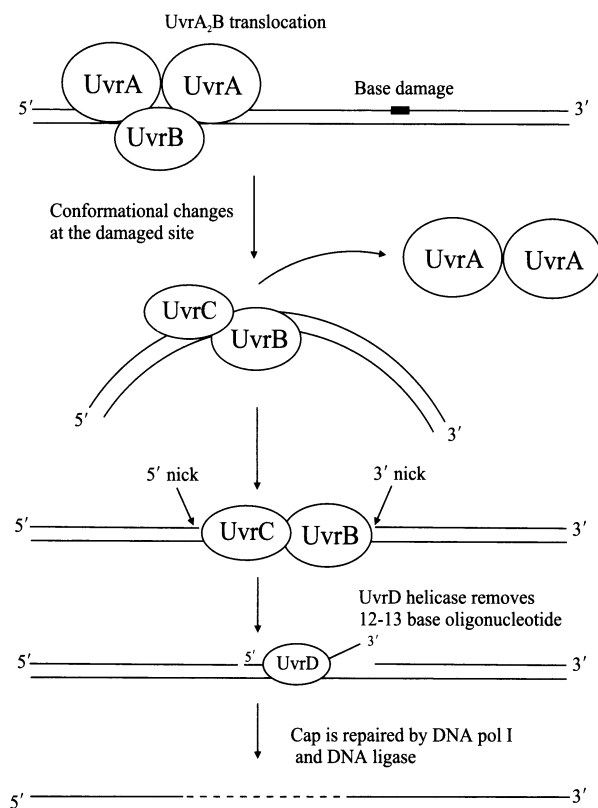


Figure 6. NER pathway in *E. coli*¹⁹⁶. UvrA₂B complex scans DNA until its movement is inhibited by the presence of bulky base damage. Initial damage recognition results in conformational changes in the UvrA₂B–DNA complex, so that UvrB binds specifically at the damaged site, and UvrA is replaced by UvrC. The binding of UvrC to UvrB causes UvrB to cut the DNA at the fourth or fifth phosphodiester bond 3' to the lesion. The UvrC protein then cuts DNA at the eighth phosphodiester bond 5' to the lesion. Once the DNA is cut, the UvrD helicase removes 12–13-nt oligonucleotide containing the lesion, and DNA polymerase I resynthesises the strand that was removed. Finally, the ligase seals the single-strand nick.

P. aeruginosa PAO1 was not inducible by DNA damage¹⁸⁸. Localisation of the promoter of the *P. aeruginosa* *uvrB* gene revealed that it lacked the LexA-binding sequence and was expressed constitutively.

Formation of a pyrimidine dimer is a common type of DNA lesion caused by UV irradiation, and can be reversed by a special type of DNA repair called photoreactivation¹⁰⁷. Photoreactivation is unique among DNA repair systems since it involves in direct reversal of the UV-induced lesion, it involves a single enzyme called photolyase which binds a pyrimidine dimer and, in a light-dependent step, monomerizes the dimer and thereafter

dissociates from the repaired lesion¹⁰⁷. The gene encoding photolyase (*phr*) isolated from *P. aeruginosa* was used to construct insertional *phr* mutants of *P. aeruginosa* and *P. syringae*¹⁰⁶. Analyses of the UV-B survival of these mutants demonstrated that under photoreactivation conditions other mechanisms such as NER and the action of *rulAB*-encoded pol V-like enzyme were also active contributors to cell survival. The fact that the expression of the *rulAB*-encoded protein increased the survival of *P. aeruginosa phr uvrA* double mutant by over 23,000-fold illustrates the importance of this enzyme whenever other DNA repair systems have not been able to remove the DNA replication-blocking damage.

6.4. Heritable Mutators in *Pseudomonas*

The efficiency of DNA repair in a cell is an important mechanism controlling the frequency of mutations. In many cases, a mutator phenotype is induced by inactivation of genes that code for DNA repair enzymes¹⁴². The most potent mutator strains in *E. coli* are those lacking either the DNA polymerase III proofreading subunit (*mutD*) or the methyl-directed mismatch repair system (*mutS*, *mutL*, *mutH*). The *mutM mutY* double mutant of *E. coli* defective for DNA repair system that corrects mistakes occurring due to the oxidation of guanine (GO repair) is as strong a mutator as *mutD*, and about an order of magnitude stronger than a strain lacking *mutS*¹⁴¹.

Most mutations are likely to be deleterious, and so the spontaneous mutation rate is generally held at a low level⁴⁹. However, a proportion of clones from natural populations of pathogenic and commensal bacteria have a strong mutator phenotype^{117, 134, 164}. By selecting a favourable allele, natural selection indirectly selects the DNA in which this mutation occurred. Consequently, mutator alleles can spread in microbial populations by hitchhiking: mutators can acquire favourable alleles more frequently than non-mutators and will therefore increase in frequency if the advantage of beneficial alleles is greater than the cost of being a mutator²³⁰. Spread of mutator alleles has been documented in *P. aeruginosa* populations infecting cystic fibrosis (CF) patients. In the study of spontaneous mutation rates of *P. aeruginosa* isolates from chronically infected CF patients, Oliver *et al.*¹⁶⁴ found that the lungs of 11 out of the 30 CF patients were colonised by mutator strains. In contrast, a mutator phenotype was not found in 75 non-CF patients acutely infected with *P. aeruginosa*. The bacterial populations chronically infecting the lungs of CF patients have to adapt to the highly compartmentalised and anatomically deteriorating lung environment, as well to the challenges of the immune defence and antibiotic therapy. Hence, severe population bottlenecks occurring after strong selective challenges enrich mutators within a population.

Subsequent studies by complementation¹⁶⁴ have revealed that seven out of eleven independent mutators studied were defective in the MMR system. Four carried mutations in *mutS*, two in *mutL*, and one in *uvrD*.

Some mechanisms facilitating the acquisition of a mutator phenotype may have been selected through evolution¹⁷⁵. Rocha *et al.*¹⁸⁹ hypothesise that inactivation of the MMR pathway might be positively selected. This argument is based on results of DNA sequence analysis indicating that the *mutS* and *mutL* sequences, in comparison with 1,000 random genes, contain larger number of direct repeats potentially capable of inducing phenotypic variability by generating deletions. They also suggest that results of the analysis are consistent with experimental evidence showing that deletions in the *mutS* gene are a major source of mutator phenotypes in natural isolates. The existence of a possible recombinational hot spot inside the *P. aeruginosa mutS* gene has also been suggested in Ref. 163. Note that the MMR not only protects against replication errors but also acts as a barrier to the recombination between moderately divergent DNA sequences. MMR-deficient cells carry out homologous recombination resulting from interspecies crosses three orders of magnitude more frequently than wild type cells^{181, 217}. Ochman *et al.*¹⁵⁹ have suggested that lateral transfer and recombinational reshuffling have played an important role in the generation of microbial diversity.

7. MUTAGENESIS IN *PSEUDOMONAS* UNDER ENVIRONMENTAL STRESS

In a growth-restricting environment (e.g., during starvation, host invasion by a pathogen), mutants arise that are able to take over the population by a process known as stationary-phase mutation⁶⁰. Many examples of stationary-phase mutagenesis in bacteria are referred in ref. [60]. Although most of the research on stationary-phase mutation has exploited *Escherichia coli*-based model systems, similar observations have also been made in other organisms including *Pseudomonas* species (many examples are listed in Table 8). One characteristic feature of stationary-phase mutations is their specific spectrum, different from that occurring among the mutants of actively growing bacteria^{61, 96, 173, 193}. This has suggested that stationary-phase mutations occur via different molecular mechanisms than mutations which arise during growth⁶⁰. Some studies suggest that starvation conditions encountered during stationary-phase incubation may permit a transient increase in the mutation rate due to variety of factors, including decreased fidelity of DNA replication and reduction of DNA repair activity^{19, 59, 213, 238}. Roth *et al.*¹⁹⁴ have proposed an alternative model that explains the occurrence of stationary-phase mutations

without requiring regulated mutability. However, SOS induction has been shown to occur spontaneously also in static bacterial populations²²⁹. Error-prone DNA polymerases pol IV, and pol V in *E. coli* are induced as part of the SOS regulon in response to DNA damage⁷². Pol IV and pol V are involved in stationary-phase mutagenesis in *E. coli*^{12, 25, 139, 263}. Bjedov *et al.*¹⁴ have demonstrated that natural isolates of *E. coli* exhibit increased mutation rates under stress encountered in aging colonies. The stress-inducible mutagenesis is genetically controlled by RpoS and the carbon-sensing regulators CyaA and CRP. Results presented in ref. [14] support the hypothesis that stress-induced mutagenesis is genetically programmed evolutionary strategy.

In addition to processes that are responsible for the generation of point mutations, the increase in transposition frequency in starving bacteria also gives a chance to generate genetic variation in a microbial population. There is evidence that starvation is accompanied by increased transposition frequency of many mobile elements^{78, 96, 113, 146, 206}. Naas *et al.*¹⁴⁹ showed that a large number of IS-related rearrangements have occurred in a 30-year-old stab, leading to a highly polymorphic population of cells. Studies of glucose-limited chemostat cultures have demonstrated that Tn5 and Tn10 can increase fitness of *E. coli*^{13, 32} whereas the fitness effect of Tn10 was associated with transposition of IS10 into new sites in the genome^{31, 32}. Upregulation of transposition of transposon Tn4652 by the host factors IHF and RpoS⁸⁹ is one of the interesting examples illustrating the involvement of transposition of mobile DNA elements in stationary-phase mutagenesis in *Pseudomonas*. Many other transpositional events discussed above in 'Involvement of transposition in mutagenesis' could also be ascribed to mechanism of stationary-phase mutations (Table 8). Interestingly, several IS-elements and transposons carry outward-directed promoters or part of promoter sequences (e.g., the -35 hexamer) that can activate silent genes^{66, 128}. In many cases, these promoter sequences have no obvious functions related to the regulation of genes internal to the element. Therefore, one should speculate that the presence of outward-directed promoters at the ends of many transposable elements hints that some of these elements may have been evolved to generate genetic variations.

Evidence supporting the idea that different mechanisms are responsible for the appearance of mutations in exponentially growing and stationary-phase cells has been found in studies using a *P. putida* test system. This test system based on promoterless phenol degradation genes *pheBA* as a reporter enabled to isolate and characterise the mutations (point mutations as well as insertions of transposable elements) accumulating in starving *P. putida*⁹⁶. The accumulation rate of Phe⁺ mutations on selective plates was found to depend on the physiological state of bacteria before the plating: The accumulation was much higher for bacteria plated from a stationary-phase culture than for those plated

from a growing culture⁹⁶. Moreover, the results of a recently published study¹⁹⁸ suggest that mutation processes in cells that have been starving for a short period are not entirely compatible with those from a prolonged starvation. It appeared that the spectrum of stationary-phase mutations among early-arising mutants differed from that of late-arising ones. The occurrence of mutations, the amount of which started to increase later (e.g., 2- to 3-bp deletions), was dependent on stationary-phase sigma factor RpoS¹⁹⁸.

The possible involvement of error-prone DNA polymerase in stationary-phase mutagenesis in *Pseudomonas* can be considered in ref. [138]. The UV response genes cloned from CAM-OCT plasmid enhanced the accumulation rate of *P. putida* catabolic mutants¹³⁸. Therefore, it is likely that the ability of cells to expand the growth substrate range may be mediated by pol V-like function encoded on CAM-OCT plasmid. Results in ref. [138] also demonstrated that the process of stationary-phase mutagenesis could be exploited to isolate the variants with potential industrial use by selecting new or improved catabolic activities in *Pseudomonas* strains. Occurrence of pol IV-dependent mutations can be detected in *P. putida* cells that have been starved several days (but not earlier) for carbon sources, and this mutagenic process is RecA-independent (R. Tegova, A. Tover, K. Tarassova, M. Tark, and M. Kivisaar, unpublished data).

The generation of strains with enhanced degradative potential in natural environment via stationary-phase mutagenesis has been demonstrated in the article by Sarand *et al.*¹⁹⁷. These authors provided evidence that *Pseudomonas* sp. strain CF600 can rapidly adapt its suboptimal ability to degrade 4-methylphenol in soil through enhancement of the ability of the pathway regulator DmpR to respond to 4-methylphenol.

Hospital isolates frequently present multidrug-resistance phenotypes as a consequence of constant selective pressure of antibiotics. The wild type *P. aeruginosa* PAO1 expresses a low level of the antibiotic efflux pump, MexAB-OprM, which gives the bacterium natural resistance to a broad spectrum of antibiotics¹⁷². Results presented in ref. [3] indicated that the emergence of multidrug-resistant mutants in *P. aeruginosa* increases under antibiotic challenge. The authors demonstrated that the incubation in the presence of tetracycline favoured the emergence of multidrug-resistance mutants expressing the efflux pump of antibiotics constitutively. Based on calculations of the number of mutants accumulating per day per viable cells in the population, the authors concluded that the mutation rate of tetracycline resistance increased by 10⁵ times after four days of incubation under tetracycline challenge.

Phenotypic switching is one of the strategies of micro-organisms for coping with environmental changes, and it is usually caused by high-frequency DNA rearrangements⁵³. In natural habitats, bacteria are living in a structured environment; they usually grow as biofilms, organised communities of cells embedded in an extracellular polysaccharide matrix and attached to a surface³⁸.

Pseudomonads are known for their striking ability to adapt to various ecological niches. *P. fluorescens* is one of the model organisms chosen for experimental studies of adaptive evolution in bacteria^{23, 24, 97, 177}. This organism has been employed in studies of the ecological and genetic causes of phenotypic divergence in microcosm populations¹⁷⁷. When selected in a spatially structured environment, populations of *P. fluorescens* rapidly diverge, producing a range of morphologically distinct niche specialist genotypes that are maintained by negative frequency-dependent selection. The radiation is driven by competition among the niche specialists. While no morphological variation can be detected in a homogeneous environment, these experiments clearly demonstrate that the phenotypic divergence is dependent upon ecological opportunity. The mutational origins of *P. fluorescens* phenotypic variants are still unknown. However, studies by Spiers *et al.*²¹⁵ have indicated that one set of the mutants forming wrinkled colonies may be caused by a mutation that alters the cell cycle regulation of cellulose-like polymer production, causing this polymer to be overproduced. The rapid adaptation to different environmental niches has been also observed in *P. aeruginosa* biofilm formation⁴⁶. The readily visible phenotypes of colony morphology mutants have been shown to be associated with mutations in regulators⁴¹. Therefore, it is possible that mutations occurring in regulatory pathways may play a general role in phenotypic switching and subsequent bacterial diversification in a structured environment.

Evidence exists that environmental signals can specifically control the rate of phenotyping switching in *Pseudomonas* species^{50, 210}. It has been shown that *P. aeruginosa* in the sputum of CF patients exists primarily as a biofilm²⁰⁹. Studies by Drenkard and Ausubel⁵⁰ have demonstrated that antibiotic-resistance phenotypic variants of *P. aeruginosa* with enhanced ability to form biofilms arise at high frequency both in vitro and in the lungs of CF patients. These variants reverted at high frequency to wild type in the absence of antibiotic in the environment. The phenotypic switching between the antibiotic-resistant and antibiotic-susceptible forms of *P. aeruginosa* in CF patients was controlled by the activity of two-component response regulator PvrR⁵⁰. The active PvrR repressed the emergence of antibiotic resistant variants and biofilm formation, whereas a mutated *pvrR* increased the frequency of appearance of resistant variants with respect to the wild type. The proportion of the population that became resistant to antibiotics through phenotypic variation was dependent on environmental conditions: The increase in the frequency of appearance of resistance variants occurred at a lower temperature, in the presence of NaCl, and under starvation. Phenotypic switching in *P. tolaasii* was also modulated by an environmentally responsive regulatory factor²¹⁰. DNA rearrangements associated with phenotypic switching between the pathogenic smooth and non-pathogenic rough forms of *P. tolaasii* occurred due to the reversible duplication of a 661-bp element within the sensor protein-encoding *pheN* locus in a RecA-dependent manner.

Some pathogenic bacteria have evolved highly mutable ‘contingency loci’ that can rapidly change due to the presence of DNA sequence repeats^{140, 147}. Hypermutable genes of pathogens encode cell surface molecules, such as adhesins or invasins involved in interactions with host molecules¹⁴⁷. Analysis of the *P. aeruginosa oprD* mutants (lacking a specialised porin protein whose inactivation causes specific antibiotic resistance) from clinical and environmental isolates indicated that similar mechanisms could play a role in the emergence of new alleles in *Pseudomonas* species as well¹⁷⁰. Whether the loci responsible for phenotypic switching in biofilm formation can also contain mutational hot spots is still unknown.

8. CONCLUSIONS

In nature, microbes are constantly confronted by variable and stressful environments. Transposition and other mutational processes have important roles in the genetic adaptation of bacteria under environmental stress (Table 8). Bacteria from the genus *Pseudomonas* are known for their ability to colonise multiple habitats and to adapt rapidly to a new environment. The involvement of transposable elements in acquisition and recruitment of genetic material in *Pseudomonas* is well documented. Also, a number of publications demonstrate that transposition can cause genetic rearrangements and affect gene activity. Some recent studies have revealed that despite its potential deleterious effect to the host cell, transposition may be up-regulated under certain environmental conditions (e.g., starvation). Other mutations occurring in stressed cells are stimulated by mechanisms that possibly involve increased mutation rates (induction of error-prone DNA polymerases, inefficiency of DNA repair systems). Although systematic studies of mutagenic processes in *Pseudomonas* are still awaiting, several new aspects have been discovered in this group of bacteria (summarised in Table 9). Also, recent papers have provided evidence that environmental signals can specifically control the rate of phenotypic switching in *Pseudomonas* species. Therefore, it is reasonable to expect that the coming years will widen our awareness of physiological regulation of mutagenic processes in bacteria.

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MOLECULAR TOOLS FOR GENETIC ANALYSIS OF PSEUDOMONADS

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1. INTRODUCTION

The genomes of several pseudomonads, including *Pseudomonas aeruginosa*¹⁶⁵, *P. putida*¹¹⁰, *P. syringae* (www.tigr.org), and *P. fluorescens* (www.sanger.ac.uk), have been entirely sequenced. To take advantage of the wealth of information revealed by these sequences and especially to reveal the role of the many *orphan* genes contained in these genomes, versatile tools are needed to gain access to their biological functions. Such tools include cloning and expression plasmids, rapid methods for cloning of large DNA segments, allele replacement vectors, tools for development of host strains for cloning and expression vectors, reporter genes for gene regulation studies, and vectors for integration of extraneous DNA (including reporter genes, regulatory elements, biosensors, etc.) into the chromosome. The growing availability of genomes facilitates genome-wide gene identification projects using insertion mutagenesis, genetic surveys of gene families involved in complex phenotypes and comparative genome analyses of different strains. Although the arsenal of genetic tools available for genetic analysis of pseudomonads has steadily grown and improved over the last decade, many of them still need improvement

to complement other modern tools available for genomic analysis, including microarray and proteomic analyses.

2. PLASMIDS

Plasmids used as genetic tools can be divided into two types, general cloning vectors and expression vectors. Basic requirements for both types of vectors include a replicon for stable maintenance of the plasmid in the respective host, a selectable marker for identification of cells containing and maintaining the plasmid, and restriction enzyme cleavage sites for cloning of DNA fragments. Because of the ease of manipulation of *Escherichia coli* and to allow for exploitation of the many reagents available for this bacterium, most cloning and expression vectors are shuttle plasmids that replicate in both *E. coli* and the respective *Pseudomonas* hosts. This is either achieved by equipping plasmid vectors with a single broad-host-range replicon that functions in *E. coli* and other bacteria or by providing two replicons, one that allows replication in *E. coli* and the other that enables replication in other hosts. For maximal versatility, cloning vectors should also contain elements that facilitate screening of recombinants and genetic elements (origin of transfer), which facilitate interspecies plasmid transfer. Expression vectors must also contain promoters, whose expression levels can be regulated and manipulated in a predictable fashion. Some expression vectors also encode affinity tags that facilitate purification of overexpressed recombinant proteins. An overview of *Pseudomonas* plasmid-based tools and accessory elements is presented in Figure 1 and representatives of various broad-host-range plasmid families are listed in Table 1.

2.1. Replicons

With the exception of integration-proficient vectors that replicate along with the host's chromosome after chromosomal integration, cloning and expression plasmids need to replicate in the respective hosts. Historically, two types of broad-host-range replicons have been popular for *Pseudomonas* vector construction, RK2 and RSF1010, because these plasmids replicate in many pseudomonads. In addition, the RSF1010 replicon is extraordinarily stable and its host range extremely broad (see Table 1 for representative vectors based on these replicons). Due to the elements necessary for replication and/or conjugal transfer, the sizes of RK2- and RSF1010-based plasmids cannot easily be reduced and the resulting cloning vectors are somewhat larger than those based on other replicons. However, the availability of complete DNA sequences for the parental plasmids^{118, 142} and a more complete understanding of their molecular biology facilitated the construction of smaller, better defined RK2- and RSF1010-based

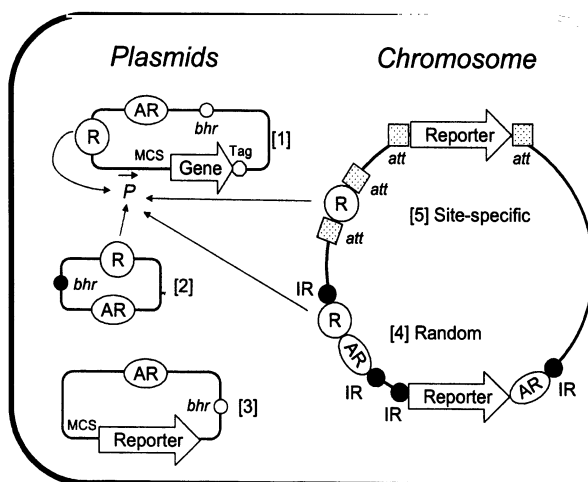


Figure 1. Overview of genetic tools available for manipulation of pseudomonads. [1] Cloning/expression vectors contain a broad-host-range replicon (bhr) and an antibiotic resistance selection marker (AR) for their selection and maintenance. The cloning of genes is facilitated by the presence of a multiple cloning site (MCS), which can be located in a gene encoding a screenable marker for visualization of recombinants, for example, by β -galactosidase activity-based blue/white screening. In expression vectors, the expression of cloned genes is directed by a promoter (P_{lac} , P_{T7} , etc.). Control of expression from these promoters can be under control of regulatory elements (R; e.g., *lac* repressor or T7 RNA polymerase) that are either located on the same plasmid [1] or on a compatible plasmid with a different *bhr* and a different AR marker [2]. These regulatory elements can also be encoded by chromosomally located sequences, either randomly integrated by transposable elements (flanked by inverted repeats, IR) [4] or site-specifically, for example, at phage attachment sites (*att*) using integration-proficient vectors [5]. Expression vectors may also encode affinity tags (Tag; e.g., hexahistidine, maltose-binding protein, FLAG) that allow affinity purification of overexpressed fusion proteins. Reporter genes (e.g., genes encoding β -galactosidase, alkaline phosphatase, luciferase, green fluorescent protein, etc.) are used for studies on gene expression, gene product localization, or for construction of biosensors. These reporters are either contained on broad-host-range plasmids that are maintained by antibiotic selection [3] or they can be randomly integrated in the chromosome via transposable elements that usually contain AR markers. Alternatively, reporter genes can be integrated at specific sites in the chromosome using integration-proficient vectors, usually at phage attachment sites. Integration vectors can be designed such that the integrated sequences are devoid of AR markers after their in vivo excision with site-specific recombinases.

vectors^{14, 17, 41, 58, 84, 96, 128, 139, 140, 181, 182}. More recently, several alternative replicons have been employed for broad-host-range vector construction. These include the replicons of pBBR1⁵, pVS1⁷⁶, and pRO1600¹¹³ and the recently discovered TFK replicon⁸⁰. All of these replicons necessitate few genetic elements for function and, when combined with small *E. coli* vectors, result in small broad-host-range cloning vectors^{30, 49, 68, 80, 89, 90, 132, 146, 147, 153, 155, 171, 176, 178}.

Table 1. Selected broad-host-range cloning and expression vectors.

Vector	Replicon	Promoter	Blue/white screening	Affinity tag	Inducer	References
pEB8	RSF1010	P _{T7}	No	No	IPTG ^a	[17]
pPLGN1	RSF1010	λ _{PR}	No	No	Temperature	[96]
pLV vectors	RSF1010	λ _{PL}	No	No	Temperature	[181]
pERD20/pERD21	RSF1010	P _m	No	No	Benzoate	[128]
pVLT vectors	RSF1010	P _{lac}	No	No	IPTG	[41]
pMMB66EH	RSF1010	P _{lac}	No	No	IPTG	[58]
pJB653 and derivatives	RK2	P _m	No	No	<i>m</i> -Toluate	[13, 14, 182]
pRK415	RK2	P _{lac}	Yes	No	IPTG ^b	[84]
pRK310 series	RK2	P _{lac}	Yes (some)	No	IPTG ^b	[51]
pLAFR5	RK2	No	No	No	—	[84]
pLAH30,31,32	RK2	P _{T5}	No	H ₆ ^d	IPTG ^a	[12]
pUCP vectors	pro1600	P _{lac}	Yes	No	IPTG ^b	[146, 153, 178]
pUCPKS/pUCPSK	pro1600	P _{T7} , P _{lac}	Yes	No	IPTG ^{a,b}	[176]
pBSP vectors	pro1600	P _{T7} , P _{lac}	Yes	No	IPTG ^{a,b}	[149]
pCom8	pro1600	P _{alkB}	No	No	DCPK ^c	[161]
pMEKm12	pro1600	P _{lac}	Yes	MBP ^e	IPTG	[101]
pSFFLAG	pro1600	P _{lac}	No	FLAG	IPTG	[27]
pME6030/6040	pVS1	—	No	No	—	[68]
pKLH series	TFK	—	No	No	—	[80]
pPP8-1	pPP8	—	No	No	—	[75]
pBBR1MCS	pBBR1	P _{T7} , P _{lac}	Yes	No	IPTG ^{a,b}	[90]
pBBad18/22	pBBR1	P _{BAD}	No	No	L-arabinose	[167]

No attempt was made to list all available cloning and expression vectors but rather to provide representative examples.
^aExpression from P_{T7} and P_{T5} requires specialized host strains containing plasmid or chromosomal copies of T7 or T5 polymerase, respectively.
^bRegulated expression from P_{lac} only possible in host strains containing a chromosomal copy of *lacI*.
^cDCPK, dicyclopropylketone.
^dH₆, hexahistidine.
^eMBP, maltose binding protein.

The type(s) of replicon(s) present on the plasmids determines their compatibility with other plasmids present in the same host cell. Plasmids are of the same incompatibility group if they cannot be stably maintained in the same cell. Conversely, plasmids from different incompatibility groups, for example, RK2 (IncP) and RSF1010 (IncQ), stably co-replicate in the same cell and can be used to co-express different genes. Most plasmids found in Gram-negative bacteria use the so-called theta-replication system, in which—with some exceptions—a specific plasmid-encoded Rep protein is necessary for replication. In contrast, most Gram-positive plasmids replicate via a rolling circle mechanism⁴⁶. A novel rolling-circle-replicating plasmid was recently isolated from *P. putida* P8 and used as a vector⁷⁵.

2.2. Multiple Cloning Sites and Screenable Markers

Multiple cloning sites (MCS; polylinkers) are a synthetic cluster of unique restriction enzyme cleavage sites that facilitate cloning of DNA fragments into plasmid vectors. To facilitate screening of recombinants, in many *E. coli* vectors these MCS are often located in DNA segments which encode screenable markers that allow visual identification of recombinants. This is possible because insertion of DNA segments into these sites disrupts the coding sequences for these markers resulting in change of phenotype. The most commonly used screenable marker involves a small segment of the *E. coli lacZ* gene, *lacZ* α ¹⁷² that permits α complementation in suitable *E. coli* hosts carrying a *lac* operon with the *lacZ* Δ M15 deletion¹²⁰. This allows visualization of a blue/white colony phenotype on media containing the β -galactosidase (β Gal) substrate 4-bromo-5-chloro-3-indolyl- β -D-galactopyranoside (XGal) in the presence of vectors carrying the *lacZ* α gene with and without inserts¹⁷². *P. aeruginosa* hosts capable of α complementation in the presence of *lacZ* α -encoding broad-host-range plasmids have been constructed by chromosomal insertion of *lacZ* Δ M15-encoding DNA fragments^{83, 153, 154}.

2.3. Selectable Markers

Cloning and expression vectors usually contain antibiotic resistance markers that allow for selection of plasmid-containing colonies after in vitro (electroporation, chemical transformation) or in vivo (conjugation) transfer of the respective plasmids into the desired host. Although many plasmids are stably maintained within their respective hosts, antibiotic selection ensures that all cells in a given population contain plasmids. The choice of marker(s) largely depends on the antibiotic-susceptibility of the respective host. Moreover, although different antibiotics may be used for selection of the same marker, they may not be applicable in all hosts. For example, whereas both

ampicillin and carbenicillin are useful for selection of the Tn3-derived β -lactamase-encoding *bla* gene in *E. coli*, only carbenicillin is useful for selection of this marker in *P. aeruginosa*. Soil pseudomonads (e.g., *P. putida*) are generally very resistant to many β -lactam antibiotics and some of them can even use penicillin as carbon source. Intrinsic antibiotic resistance often hampers genetic analysis of many pseudomonads. Antibiotic resistance markers useful for broad-host-range vector construction may be obtained as cassettes from various sources^{2, 4, 49, 155} or by PCR amplification of the respective coding sequences. Besides antibiotic resistance or biocide-resistance⁷⁸ (e.g., triclosan) markers, metabolic markers may also be useful in certain applications where selection with antimicrobials is less feasible. The *E. coli* lactose operon⁸⁷ can be utilized as a selectable marker in pseudomonads, many of which are naturally devoid of lactose utilization genes. Pseudomonads carrying specific siderophore receptors can be isolated and selected from environmental samples using iron-deprived medium supplemented with the appropriate siderophore¹²³.

2.4. Methods for Plasmid Transfer

Although most plasmids can be readily introduced into many pseudomonads by transformation (chemical^{24, 113} or electroporation^{47, 86, 101}), conjugation of large plasmids is still a widely used method of transfer and for some applications more efficient than other transfer methods. Therefore, many broad-host-range plasmids contain an origin of conjugal transfer, usually the *oriT* of RK2 or the *mob* region of RSF1010. Cassettes containing the *oriT* have been derived and serve as a ready source of *oriT*-containing DNA fragments for vector construction^{3, 155}. Because many broad-host-range plasmids contain minimal *oriT* sequences (~250 bp), they are incapable of self-transfer and the mobilization functions need to be provided in *trans*. For this purpose, *E. coli* mobilizer strains SM10 or S17-1 have been constructed, from which plasmids can be efficiently mobilized because they contain an integrated copy of RP4 that supplies the needed transfer functions in *trans*¹⁵⁸. Alternatively, a triparental mating system can be used in which *E. coli* containing plasmid pRK2013, a ColE1-based plasmid carrying the *tra* genes of RK2, is employed to transfer the mobilizable plasmid from a separate donor strain to a recipient strain^{55, 135}.

Many broad-host-range plasmids contain *cos* sites, which are short regions of DNA containing the cohesive ends of *E. coli* phage λ . Such vectors are referred to as cosmids and are capable of accepting large (30–48 kb) DNA fragments. After ligation, cosmids can be efficiently packaged in vitro into λ particles and used to transfect *E. coli*. From there, they can be conjugally transferred to pseudomonads^{34, 36, 38, 84, 135}.

2.5. Expression Vectors

To achieve regulated expression of cloned genes in pseudomonads, many broad-host-range cloning vectors contain promoters whose activities can be regulated. These include: the *E. coli lac* operon promoter (P_{lac}) or its derivatives (P_{tac} and P_{trc})¹⁷⁵ repressible by the *lacI*-encoded *lac* repressor and inducible by inducers such as isopropyl- β -D-thiogalactopyranoside (IPTG)^{41, 58, 62, 153, 178}; the *E. coli aroBAD* operon promoter (P_{BAD}) repressible by AraC and inducible by L-arabinose^{63, 111, 167, 186}; the T7 promoter (P_{T7}) from the T7 bacteriophage gene 10, which requires expression of the T7 RNA polymerase (RNAP) gene, generally under control of the P_{lac} -*lacI* system^{35, 40, 153}; the T5 promoter (P_{T5}) system under control of the P_{lac} -*lacI* system¹²; the rightward (λ_{PR}) and leftward (λ_{PL}) promoters of bacteriophage λ under control of a temperature-sensitive λ repressor^{96, 181}; P_{alkB} from *P. putida* (*oleovorans*) under positive control of AlkS and inucible by various alkanes¹⁶¹; P_m and P_u from the *P. putida* TOL plasmid pWWO that are under control of XylR and XylS, and inducible by xylenes and toluene (P_u /XylR), and alkylbenzoates (P_m /XylS), respectively^{13, 14, 182}.

Of these examples, the *lac* operon-based promoters and P_{T7} are probably the easiest to apply in pseudomonads because the biology of these two systems is well understood. *P. aeruginosa* strains serving as hosts for regulated expression from *lac* operon-based promoters and P_{T7} were derived by chromosomal integration of the respective regulatory and expression elements^{40, 44, 70, 153}. Similar *P. putida* strains have also been engineered using a mini-Tn5 chromosomal insertion system as the carrier of the T7 RNAP gene⁷⁰. The genes coding for Lac repressor and T7 RNAP can also be provided from compatible broad-host-range plasmids^{12, 17, 35, 70}.

2.6. Affinity Tags

To facilitate purification of recombinant proteins in pseudomonads, broad-host-range vectors were developed that allow regulated overexpression of fusion proteins with affinity tags. Vectors expressing proteins containing hexahistidine tags were used to overexpress and purify proteins from *P. putida*^{12, 20} and *P. aeruginosa*¹⁵³ using nickel affinity chromatography. A broad-host-range vector encoding the maltose-binding protein (MBP) fusion system was used for production of a non-ribosomal peptide synthetase in *P. syringae*. MBP fusion proteins can be purified by amylase affinity chromatography^{50, 102}. Vectors for expression of FLAG-tagged proteins were designed and used to purify FLAG fusion proteins from *P. syringae* by immunoaffinity chromatography²⁷.

2.7. Host Strains for P_{lac} and P_{T7} containing Plasmids

To achieve regulated expression from the P_{lac} and P_{T7} promoters, *Pseudomonas* host strains must contain the appropriate regulatory elements either located on compatible plasmids^{12, 17} or stably integrated into the chromosome^{44, 70, 83, 153}. Using mini-CTX-based integration-proficient vectors, *P. aeruginosa* host strains were constructed that allow regulated expression from P_{lac} and P_{T7} by integrating $lacI^q$ - P_{lac} - $lacZ\Delta M15$ and $lacI$ - P_{lacUV5} - $T7$ RNAP cassettes, respectively¹⁵³. Because both of these cassettes are unmarked and are incorporated at the neutral ϕ CTX attachment site they neither compromise any antibiotic markers used on cloning vectors nor do they affect the fitness of the host strains. An alternative way to engineer an inducible T7 RNAP system in the chromosome is the assembly of the RNAP gene under the control of a benzoate-responsive promoter inside the boundaries of a mini-Tn5 transposon, which can be inserted at ease in any target strain⁷⁰.

3. METHODS FOR IN VIVO CLONING OF *PSEUDOMONAS* GENES

Despite the elucidation of genomic sequences for various pseudomonads, including *P. aeruginosa*¹⁶⁵, *P. putida*¹¹⁰, *P. syringae* (www.tigr.org), and *P. fluorescence* (www.sanger.ac.uk), which greatly facilitates PCR-based cloning of DNA sequences, the cloning of larger DNA fragments, for example, those containing pathogenicity islands or lipopolysaccharide biosynthetic gene clusters¹³⁰, require methods that allow the rapid cloning of such large DNA fragments. A phage D3112-based method was described for *P. aeruginosa*³² and used successfully for the cloning of several chromosomal DNA segments^{23, 32, 143}. With this method, mini-D3112 replicons are employed that incorporate a broad-host-range origin of replication and an antibiotic resistance marker between the ends of the transposable phage D3112. These plasmids are then transformed into a strain harboring a temperature-sensitive D3112 prophage. A mixed lysate is obtained by temperature induction and used to transduce an appropriate *P. aeruginosa* recipient strain. Recombinant plasmids are identified as drug-resistant colonies that simultaneously receive the gene(s) of interest, which must confer a selectable or screenable phenotype. The gene(s) of interest can then be subcloned into other suitable cloning and/or expression vectors.

The one limitation of this procedure is that it can be used only in *Pseudomonas* strains sensitive to the D3112 phage, an exceptional situation rather than a rule. An interesting procedure to clone in vivo genes from soil and rhizosphere *Pseudomonas* is the exploitation of the phenomenon called

retrotransfer¹³⁴. This is based on the property of some large plasmids of *Pseudomonas* (for instance, pWW0, encoding catabolic genes) and other species, which travel from a host strain to a recipient and then return to the original host, bringing with it DNA from the recipient. The resultant host strain with DNA from the recipient is called a retrotransconjugant, from which the desired insert can subsequently be retrieved.

A more general approach involves yeast targeted recombinational cloning to isolate large chromosomal segments^{130, 131} (Figure 2). This method exploits the yeast (*Saccharomyces cerevisiae*) recombination pathway as a tool for sequence-specific assembly of recombinant plasmids. Targeted segments are amplified from bacterial genomic DNA by using tailed primers that create overlaps with yeast recombinational vector sequences. Plasmids are then assembled in a single step by combining a yeast-*E. coli* shuttle vector containing a *URA3* yeast selectable marker (plus a bacterial antibiotic resistance marker and plasmid replicon), the two PCR-amplified targeting segments

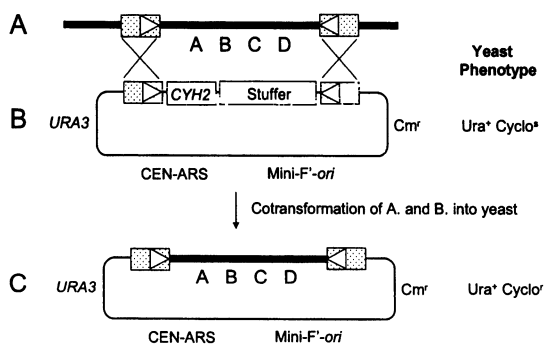


Figure 2. Cloning of *Pseudomonas* chromosomal DNA fragments by employing yeast recombinational vectors. A. Schematic illustration of the chromosomal region targeted for cloning with the location of sequences of the ~500 bp targeting fragments (indicated by the stippled boxes). B. *E. coli*-yeast (*S. cerevisiae*) shuttle plasmid with the cloned 500 bp targeting fragments separated by a stuffer fragment and the yeast *CYH2* gene that confers a negative dominant cycloheximide resistant (*Cyclo*^r) phenotype on the cycloheximide sensitive (*Cyclo*^s) yeast host. The plasmid also contains yeast markers *URA3* and CEN-ARS, which allow plasmid selection and segregation-replication, respectively, in yeast. Replication and selection in *E. coli* is provided by the origin of replication from the F plasmid (Mini-F'-ori) and a chloramphenicol resistance marker (*Cmr*). C. Cotransformation of the shuttle plasmid and sheared *Pseudomonas* DNA fragments, including the fragment depicted in A., into yeast results in recombinational cloning of the targeted fragment. The recombination event deletes the *CYH2* gene and the stuffer fragment from the shuttle plasmid and confers a *Cyclo*^r phenotype on the yeast host (phenotypes conferred by the shuttle plasmid and the recombinant plasmid, respectively, are indicated on the right side of the figure. After initial characterization in yeast, the recombinant plasmid can then be transformed into *E. coli* for further characterization.

(~500 bp each), and a central fragment that carries the yeast *CYH2* gene (a counterselectable marker conferring dominant cycloheximide sensitivity to the cycloheximide-resistant [cyclo^r] yeast host strain used for recombinational cloning) and a stuffer fragment. For recombinational cloning, genomic DNA is mechanically sheared and co-transformed with linearized recombinational cloning plasmid into the yeast host strain. Ura⁺ Cyclo^r transformants contain mostly recombinant plasmids from which the *CYH2* gene was lost by recombination with a genomic segment. Plasmid DNA from desired recombinants is then recovered and transferred to *E. coli* for further characterization. This technology was used to clone the LPS O-antigen biosynthetic loci from 20 different *P. aeruginosa* serotypes¹³⁰.

4. METHODS FOR GENOMIC INTEGRATION OF DNA FRAGMENTS

Genomic integration of DNA fragments is advisable in situations where maintaining the genes of interest in a monocopy dose is desirable or when plasmids cannot be readily retained. These cases include fine studies on regulation of given promoters, construction of biosensors destined for environmental release, studies of bacterial biofilms, or genetic engineering of bacteria for bioremediation. Various molecular tools were developed that allow delivery of genetic material and stable integration into the chromosome¹⁴⁹. The two most generally applicable methods for single-copy gene delivery into the genome of pseudomonads include the use of transposons and integration-proficient vectors.

4.1. Transposons

The perhaps most commonly used method for single-copy chromosomal insertion of genes in pseudomonads involves mini-Tn5 derivatives, which readily transpose in most bacteria after conjugal delivery via suicide vectors^{39, 42}. This technology has found widespread applications and has been used for incorporation of reporter genes for gene regulation studies¹⁷⁹, engineering of strains for bioremediation^{25, 170}, construction of biosensors^{65, 66, 177}, tracking of genetically engineered bacteria in the environment^{31, 127}, analysis of biofilms²², and construction of host strains for regulated expression from various plasmid promoters⁴⁴. The advantages of the mini-Tn5-based technology include their applicability to a wide-range of bacteria, the possibility to make several insertions in the same strain and the choice of a wide range of antibiotic and non-antibiotic selection markers⁴³. These markers include resistances

to a number of heavy metals and metalloids such as arsenite, mercury, or tellurite^{43, 137}, which pose little environmental risk when present in strains destined for environmental release. However, mini-Tn5 insertions occur randomly in the genome and position effects cannot easily be controlled. A simplified version of the mini-transposon approach involves the generation in vitro of the transposition synaptic complex (transposome) with the desired gene and the transformation of the target strain⁶¹(see Section 7 later).

Another development that makes the mini-transposon system a favorite choice for chromosomal placing of cloned genes is the possibility to excise the selection markers following its insertion in the chromosome. Previous work on the multimer resolution system (*mrs*) of broad host range plasmid RP4 had shown that DNA segments flanked by tandem *res* sites and cloned in a multicopy vector could be precisely deleted in vitro and in vivo by the product of the *parA* gene. Based on these observations, the *mrs* system has been exploited to develop a general method that permits the precise excision of antibiotic resistance markers present in mini-transposon vectors after the transposition event has taken place^{91, 117}. This is based on site-specific recombination between two directly repeated 140-bp resolution (*res*) sequences of RP4 catalyzed by a resolvase encoded by the *parA* gene, which is provided by a conditional replication plasmid. This strategy permits the stable inheritance of heterologous DNA segments virtually devoid of the sequences used initially to select their insertion. The mechanism of multimer resolution (which works over 60 kb in RP4), makes this strategy generally applicable to DNA fragments of virtually any size, even large chromosomal segments. The ParA-*res* system is particularly suited to generate chromosomal insertions of heterologous DNA segments required for metabolic engineering of strains destined for environmental release¹¹⁷. Excision of chromosomal markers can also be achieved using the site-specific recombination system based on yeast FIp recombinase (flippase)^{71, 105, 145}.

A transposable phage D3112-based method was described for random, single-copy insertion of DNA into the *P. aeruginosa* chromosome but its application is limited to this bacterium^{83, 154}.

4.2. Integration-Proficient Vectors

A second approach to heterologous gene integration involves the usage of integration-proficient vectors. These vectors integrate at defined, naturally evolved, or engineered sites within bacterial genomes and therefore they do not generally affect the host's fitness. There are various methods to achieve site-specific integration in the genomes of pseudomonads, mainly vectors with phage attachment sites and Tn7-based vectors. In other cases, the basis of the integration relies on the use of molecular assets recruited from bacterial integrons or from yeast site-specific recombination systems.

4.2.1. The *P. aeruginosa* mini-CTX System. The integration-proficient mini-CTX vectors (Fig. 3) were specifically developed for site-specific integration into the *P. aeruginosa* chromosome⁷². These phage CTX-based integration-proficient mini-CTX vectors allow insertion of gene cassettes at a defined location, the 30-bp *attB* sequence⁶⁷, which is located at 2.94 Mb on the 6.24 Mb strain PAO1 chromosome¹⁶⁵. The *attB* site is a phage attachment site that has naturally evolved to allow phage integration without causing a mutant phenotype and without compromising fitness. Following site-specific vector integration at the chromosomal *attB* site, which is catalyzed by the ϕ CTX integrase encoded by the vector, unwanted sequences, including plasmid-associated promoters and antibiotic markers, can be removed *in vivo* by yeast Flp recombinase⁷¹. Using a series of PCR primers, integration at the *attB* site and subsequent Flp-mediated deletion of unwanted plasmid sequences can be easily monitored by colony PCR^{72, 187}. Specialized mini-CTX vectors have been used to construct *P. aeruginosa* host strains allowing regulated expression from the T7 and *lac* promoters, as well as for studying gene expression using *GFP*-, *lac*- and *lux*-based reporter genes^{150, 153}. The mini-CTX vectors have proven very useful for studies of gene expression in *P. aeruginosa* biofilms¹⁸⁷. The main drawback of the mini-CTX series of integration vectors is that their

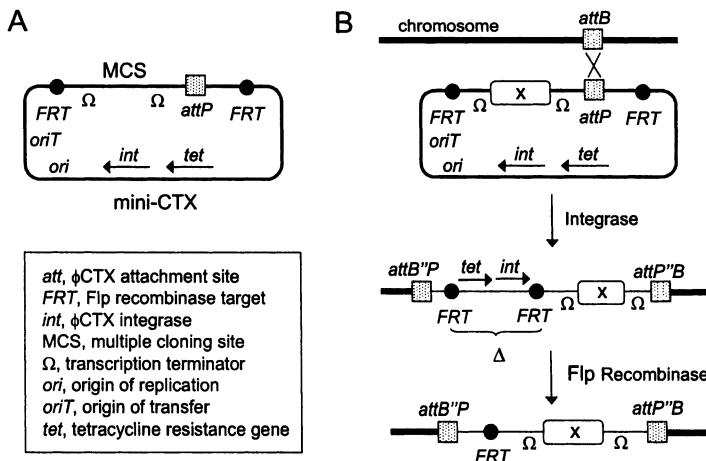


Figure 3. The mini-CTX vector system for chromosomal gene integration in *P. aeruginosa*. A. Map of a mini-CTX vector and its pertinent features. B. Integration of genetic element X at the *attB* locus, which is catalyzed by integrase and selected as tetracycline-resistant colonies. The bracketed sequences are subsequently deleted *in vivo* by the action of Flp recombinase, resulting in a strain that is devoid of antibiotic resistance markers and other undesired plasmid sequences. This system can be used for the chromosomal integration of regulatory elements, reporter gene fusions, or other genetic traits.

applications are confined to *P. aeruginosa*. They do not integrate even in bacteria with nearly identical *attB* sites, for example, *P. putida* strain KT2440, whose genome contains an *attB* site that is located in a tRNA^{Ser} gene and within the same genomic organization as that found in *P. aeruginosa*, and which over 30 bp is 86% identical to *P. aeruginosa attB*¹⁵³.

4.2.2. Tn7-Based Site-Specific Integration Vectors. Whereas most transposable elements move at low frequencies and insert into many different target sites, displaying little target site-selectivity, Tn7 is distinguished by its ability to insert at a high frequency into bacterial chromosomes with a defined orientation at a specific insertion site, *attTn*^{729, 45}. Tn7 attachment sites are found in the chromosomes of many bacteria^{28, 86} and are located downstream of the 3' ends of the respective *glmS* genes, encoding a cell wall biosynthetic enzyme. It is noteworthy that there is no discernable homology between points of insertion in the different bacteria. However, for *E. coli* it has been well established that the sequences required for Tn7 insertion extend into the 3' end of the *glmS* coding sequence, which is highly conserved in different bacterial species, indicating that site-specific Tn7 insertion reflects utilization of its transposase pathways and substantial conservation of the *glmS* gene²⁸. Since the transposon itself is inserted downstream of *glmS* in the intergenic region with the downstream gene, its insertion does not affect expression of either gene^{29, 45, 107}. Transposition of Tn7 minimally requires the Tn7 left (Tn7L) and Tn7 right (Tn7R) ends, and the transposase complex, which comprises the products of five genes, *tnsABCDE*²⁹. Several Tn7-based mini-transposons were designed and used in different pseudomonads for reporter gene construction^{74, 86, 156}. Some of these mini-Tn7s contain less than 200 bp of Tn7L and Tn7R, and transpose at relatively high frequencies (10^{-3} to 10^{-4}) in the presence of a helper plasmid transiently expressing the transposase⁶. Wild-type Tn7 transposes efficiently in *P. aeruginosa*¹⁸. We recently developed several mini-Tn7 vectors with removable selection markers for use in pseudomonads and determined their precise insertion site downstream of *glmS* in *P. aeruginosa* (K.-H. Choi and H.P. Schweizer, manuscript in preparation).

4.2.3. Integration with Site-Specific Recombination Systems. As mentioned above, the yeast flippase (Flp) is a site-specific recombinase, which acts on short (68 bp) sequences named Flp recombinase target (*FRT*) sites with a very high efficiency. The combination of *FRT* sites with transient expression of the flippase in bacteria is increasingly being recognized as a useful asset for vector development^{71, 105, 145}. Some of these vectors facilitate the integration of a single *FRT* site into a target chromosome by homologous recombination. A matching vector contains two *FRT* sites that bracket a modified multiple cloning region for DNA insertion. After integration, a helper plasmid expressing the Flp

recombinase allows precise *in vivo* excision of the replicon and the marker used for selection. Sites are also available for temporary insertion of additional functions, which can be subsequently deleted with the replicon. Only the DNA inserted into the multiple cloning sites (passenger genes and homologous fragment for targeting) and a single *FRT* site remain in the chromosome after excision. With these vectors, a single antibiotic selection system can be used repeatedly for the successive improvement of strains with precise deletion of extraneous genes used during construction⁷¹. Although not devised originally for *Pseudomonas*, this approach will undoubtedly be of widespread use in many Gram-negative bacteria. Similarly, some elements of bacterial integrons have been exploited for designing vectors capable of delivering DNA segments or even whole plasmids to the chromosome of a target strain. For instance, conditional replication plasmids containing the *int* gene and the recombination hot spot 2 (RHS-2) from the integron in Tn21 were constructed to obtain Tn21 integrase (Int21)-mediated plasmid integration a target chromosome. Interestingly, the resulting colonies bore the plasmid specifically integrated through the RHS into different positions of the chromosome. Integration sites in the chromosome were pentanucleotides with the sequence described for Int21 secondary sites^{56, 57}.

5. REPORTER GENES

Reporter genes are useful to study gene expression and they also allow genetic marking of bacteria so that they can be easily tracked and identified. Transcriptional fusions result from placing a promoterless reporter gene under the transcriptional control of the gene(s) under study and thus provide information about genetic control mechanisms governing transcription of these genes. Translational fusions result from in-frame fusions of promoterless reporter genes lacking their own transcription and translation initiation signals to target genes providing both of these signals. The resulting hybrid proteins can provide information on posttranslational events, including protein localization. The use of various bacterial reporter genes and their assay formats have recently been reviewed^{34, 141, 149}. Reporter gene fusions in pseudomonads can be obtained by cloning fragments into plasmids containing promoterless reporter genes^{54, 93, 133, 147, 150, 151, 157}, by transposition of transposons carrying promoterless reporter genes⁴⁴ or by homologous recombination with the chromosome⁷⁹.

A widely used reporter gene is *lacZ* from the *lac* operon of *E. coli*, which encodes β -galactosidase. Many vectors carrying a promoterless *lacZ* gene have been used for constructing gene fusions in various pseudomonads^{54, 79, 85, 93, 133, 139, 147, 150, 151, 157}. Although the *lacZ* α fragment was also used as a reporter in *P. aeruginosa*, it requires specially designed

bacteria that carry the *lacZ*ΔM15 allele¹³². Other promoterless indicator genes that have been used in various pseudomonads include *xylE*, catechol-2,3-dioxygenase from *P. putida*^{81, 88, 135, 148}; *galK*, galactokinase from *E. coli*³⁷; *inaZ*, ice nucleation activity^{100, 109}; *luxAB* or *luxECABD* from *Vibrio fischeri* and *Xenorhabdus luminescens*, respectively^{42, 53, 150}; *luc*, firefly luciferase¹³⁹; *phoA*, alkaline phosphatase from *E. coli*^{16, 53, 168}; and GFP, green fluorescent protein from *Aequorea victoria*^{22, 64, 106, 109, 116, 166}. Promoterless antibiotic resistance genes can also be used as reporters^{53, 54, 104}. Of the many reporter genes mentioned above, those encoding β-galactosidase (*lacZ*), luciferase (*lux* or *luc*), and green fluorescent protein (*GFP*) are now most widely used. The choice of reporter gene and assay method ultimately depend on the purpose of the experiment, and their applications are only limited by the availability of delivery systems and possible interference by endogenous host enzymes or intrinsic antibiotic resistance.

Surface reporters are distinct types of reporter systems that involve an indirect immunological detection of promoter activity. This is based on the production of a reporter epitope expressed on the surface of bacterial cells. Typically, an antigenic sequence is inserted by recombinant means in permissive regions of an outer membrane protein (OMP; e.g., the LamB protein of *E. coli*). When the hybrid OMP gene is transcribed, the epitope becomes presented on the surface of the bacterial cells in a configuration available to specific antibodies¹⁹. Unlike other procedures used so far to follow the activity of bacterial promoters, this indicator system relies on a physical property of the reporter product (its recognition by a cognate antibody) instead of an enzymatic or optical trait. This allows also the immunomagnetic separation of cells within a population, which express a given promoter at a given time⁴³. The LamB-A6 protein has been used to examine the activity of TOL plasmid promoters *in situ* for biodegradation of toluene with an anti-A6 epitope monoclonal. A similar approach using a hybrid PhoE-ColA as a reporter was successful, with some limitations, to detect activity *in situ* of promoters of the plant root-colonizing strain *P. putida* WCS358 with an anti-colicin A monoclonal antibody¹⁸⁸. Also, the OprF porin of *P. aeruginosa* has been found to contain permissive sites eligible for genetic insertion of heterologous peptides¹⁸⁴, thus providing an additional OMP amenable to being developed as a surface reporter.

6. GENE REPLACEMENT METHODS

Replacement of wild-type alleles on the chromosome with plasmid-borne mutations requires (a) a method for transfer of mutant alleles into the bacterium, (b) integration of the mutant allele into the chromosome by homologous

recombination in place of the wild-type allele, preferably with concomitant loss of plasmid sequences, and (c) means for detection and verification of the mutant¹⁶⁹. Plasmid-borne mutant alleles of a gene or operon are either generated by changing individual nucleotides by site-directed mutagenesis, by insertional inactivation at a unique site within the gene, or by deletion of segments from the gene. The latter two are most often achieved by insertion of antibiotic resistance makers, thus “marking” the mutation. In any case, the mutated plasmid-borne sequences have to be transferred (most often by conjugation) into the recipient bacterium. Since the gene replacement vectors are suicide vectors, exconjugants result from chromosomal integration via Campbell-type homologous recombination to form a merodiploid (Figure 4). Several types of suicide vectors have been used for gene replacement in pseudomonads (Table 2). They either contain narrow-host-range or conditional replicons. They mainly differ by the absence or presence of counterselectable markers. To aid the excision of unwanted DNA sequences from merodiploids, newer

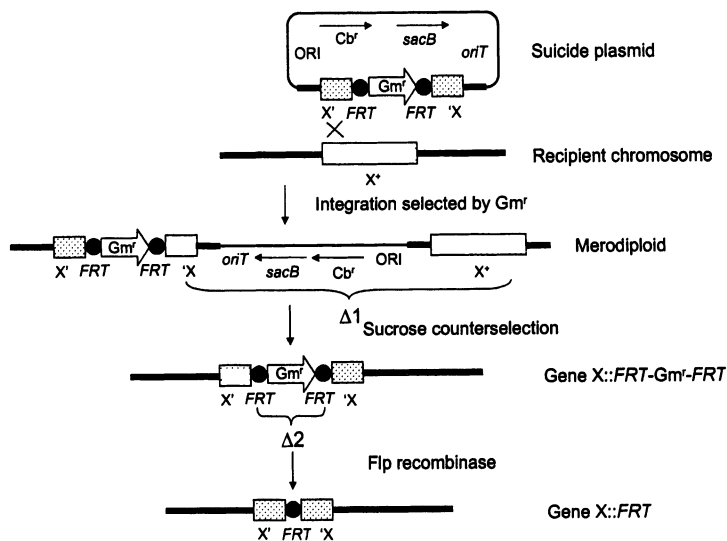


Figure 4. A common strategy for allele replacement in pseudomonads. The mutated gene sequences *X* (here shown tagged with a gentamycin resistance [*Gm^r*] marker that is flanked by Flp recombinase target [*FRT*] sites) are cloned in a mobilizable suicide plasmid. This recombinant plasmid is conjugally transferred to a recipient where it integrates into the chromosome via homologous recombination, thus yielding a merodiploid. Resolution of the merodiploid state by deletion of the bracketed sequences marked $\Delta 1$ is promoted by sucrose counterselection, resulting in a mutant chromosome containing an antibiotic resistance marker. If desired, the antibiotic resistance marker can be deleted (marked $\Delta 2$) by Flp recombinase, yielding an unmarked mutant strain. Abbreviations: *Cb^r*, carbenicillin resistance marker; *oriT*, origin of transfer; *ORI*, narrow-host-range ColE1 origin of replication; *sacB*, *Bacillus subtilis* gene encoding levansucrase.

Table 2. Selected gene replacement vectors for *Pseudomonas*.

Vector	Replicon	MCS ^a	Blue/white screening	Selection against vector	Special requirements	References
pSUP series	ColE1 or p15A	No ^b	No	None	None	[158]
pGP704	R6K	Yes	No	None	None	[108]
pKAS series	R6K	Yes	No	Yes (via dominant <i>rpsL</i> ⁺ allele)	Works only in <i>rpsL</i> strains ^c	[159]
pRTP1	ColE1	No ^b	No	Yes (via dominant <i>rpsL</i> ⁺ allele)	Works only in <i>rpsL</i> strains ^c	[163]
pKGN101	R6K	Yes	No	Yes (via <i>sacB</i> -mediated sucrose-sensitivity)	None	[82]
pCVD442	R6K	No ^b	No	Yes (via <i>sacB</i> -mediated sucrose-sensitivity)	None	[52]
pEX series	ColE1	Yes	Yes	Yes (via <i>sacB</i> -mediated sucrose-sensitivity)	None	[71]

No attempts were made to list all suicide plasmids available for allele replacement in pseudomonads but representative derivatives of the major types of vectors are listed.

^aMCS, multiple cloning site.

^bThese vectors contain multiple unique sites for cloning of DNA fragments but they are not clustered in an MCS.

^c*rpsL* strains are streptomycin resistant.

generations of gene replacement vectors were equipped with counterselectable markers.

Vectors containing the *sacB* gene from *Bacillus subtilis* confer sucrose-sensitivity and therefore selection of sucrose-resistant derivatives selects for the desired excision event^{71, 144, 152}. These gene replacement systems can also be used to introduce unmarked deletion or single-base substitutions (e.g., temperature-sensitive alleles⁹²) into the chromosome but resolution of the merodiploid will only lead to replacement of the wild-type allele in a portion of the sucrose-resistant colonies, whereas a significant number of colonies will revert to wild-type. The use of a suicide plasmid and sucrose counterselection for allele replacement is illustrated in Figure 4. Some selected allele replacement vectors used in various pseudomonads are listed in Table 2. The pSUP plasmids found

widespread applications, including *P. aeruginosa*^{112, 164}. The pEX gene replacement vectors originally developed for *P. aeruginosa*^{71, 144, 152} were successfully applied in other bacteria, including several that are closely related to pseudomonads, for example, *Bukholderia cepacia*⁹⁹ and *Stenotrophomonas maltophilia*¹⁸⁹. Other vectors used to obtain *P. aeruginosa* mutants with the assistance of sucrose counterselection are pKNG101¹⁸³ and pCVD442¹²⁹. Suicide plasmid pGP704 was applied in *P. aeruginosa*¹⁶⁹ and *P. putida*¹⁸⁰, and pRTP1 in *P. aeruginosa*⁵⁹.

The *B. subtilis* *sacB* gene is the most widely used counterselectable marker because in principle it works without the necessity for certain background mutations. Yet, it may not be optimal in all cases and novel counterselection strategies are always sought. For instance, vectors containing the wild-type *rpsL* gene confer streptomycin susceptibility in a *rpsL* mutant background and this marker can therefore also be used to counterselect for unwanted DNA sequences^{160, 163}. Because streptomycin resistant *P. aeruginosa* mutants are easily isolated, the method has been used for isolation of mutants in this bacterium⁵⁹. Another alternative counterselection procedure involves the use of the *amiE* gene of *P. aeruginosa*, the product of which (an aliphatic amidase) converts fluoroacetamide to the toxic compound fluoroacetate²⁶. In some cases, direct selection of double recombination events is possible through the reconstruction of an otherwise inactive antibiotic resistance gene assembled with sequences bearing *supF*-suppressible codons⁸⁵.

The antibiotic resistance marker(s) chosen for isolation of chromosomal insertion or deletion mutations can be excised from the chromosome in vivo by Flp recombinase, which allows recycling of the antibiotic resistance cassette used in the gene replacement experiment^{71, 72}. Because every Flp-mediated excision leaves behind a Flp recombinase *FRT* site, care must be taken not to select chromosomal rearrangements. Such rearrangements occasionally do occur but they are extremely rare and can easily be detected by PCR or genomic Southern analyses⁷. Although the broad-host-range Flp-*FRT* system was originally developed mainly for use in *P. aeruginosa*⁷¹, it has also successfully applied to *P. putida* (H.P. Schweizer, unpublished observations). The *mrs* from the broad-host-range plasmid RP4 was used to generate site-specific deletions in transposons integrated into the *Pseudomonas* chromosome designed to specifically delete the resident antibiotic resistance markers⁹¹. The removal of antibiotic resistance genes is desirable for the engineering of strains destined for environmental release¹¹⁷ or for construction of attenuated, live vaccine strains¹²².

A drawback of conjugal transfer of suicide plasmids during gene replacement analyses is the almost inevitable merodiploid formation during the Campbell-type homologous recombination between plasmid-borne and chromosomal sequences. To circumvent merodiploid formation, a "recombination-aided" procedure was developed for *P. aeruginosa*¹⁸⁵. The *P. aeruginosa*

recipient contains a plasmid expressing the I-*Sce*I endonuclease, which excises the fragment destined for homologous recombination from a conjugally transferred suicide plasmid because it is engineered to contain the recombination fragment between two I-*Sce*I sites. The resulting linear DNA fragment then recombines very efficiently with the host's chromosome resulting in allele replacement.

7. IMPROVED METHODS FOR INSERTIONAL MUTAGENESIS

Any *wet* analysis of a given strain of defined phenotype starts with the generation of mutants, and transposons in their various forms remain the molecular tools of choice for such analyses. The use of transposons, mini-transposons and plasposons for genetic analysis of *Pseudomonas* has been reviewed several times in recent years^{43, 44, 48, 138} and is not discussed in detail here. In this chapter we focus on a few improvements that expand the range of applications of such pivotal genetic assets.

Until very recently, DNA transpositions have usually involved strictly *in vivo* approaches, in which the transposon of choice and the gene encoding the transposase responsible for catalyzing the transposition have to be introduced into the cell to be studied. However, all *in vivo* systems have a number of technical limitations. For instance, the transposase must be expressed in the target host, and the transposon must be introduced into the host on a suicide vector. A number of *in vitro* transposition systems have been developed more recently, which bypass many limitations of *in vivo* systems. One significant development in this regard has been the development of a novel procedure to transform target strains with a synaptic Tn5 transposition complex preformed *in vitro*^{61, 73}. In the most used form, such a complex ("transposome") contains a purified and hyperactive Tn5 transposase, along with a PCR fragment bearing a selection marker and/or another DNA segment and flanked by optimized Tn5 ends. This combination generates a circular (but not closed) complex, which can then be readily transformed into electrocompetent cells, followed by selection for the resistance marker encoded by the transposome. This procedure avoids the time-consuming mating steps associated with the delivery of mini-transposons *in vivo*, and eliminates the formation of co-integrates with the delivery plasmid. However, purification of the transposase is a tedious process and the alternative commercial product (www.epicentre.com) is relatively expensive. However, because of the ease of assembling transposomes *à la carte* with PCR fragments, they are probably destined to replace mini-transposons altogether in the not too distant future.

A second development for improved insertion mutagenesis is the exploitation of eukaryotic transposase systems *in vivo* and *in vitro*. For instance, the *mariner* family transposons are widespread among eukaryotic organisms (e.g., *Drosophila*) and have the property of transposing at extremely high frequencies⁹⁴ in the absence of added cofactors or host cell factors¹³⁶. On this basis various transposition systems have been developed with mini-transposons consisting of short inverted repeats flanking antibiotic resistance markers. These elements efficiently transpose after expression of the transposase from an appropriate bacterial promoter. But in reality, the main value of the *mariner* system for *Pseudomonas* is the possibility to set up the transposition reaction *in vitro*¹⁸⁵ followed by a massive recombination of the insertions in the target strain. In one case, a highly efficient *mariner* transposition system was used to mutagenize *P. aeruginosa* cosmids *in vitro* and combined with an efficient allelic exchange system by using the *I-SceI* homing endonuclease to generate mutants within targeted regions of the chromosome for genetic footprinting analyses. This technique (genetic footprinting) is so efficient that chromosomal regions that do not contain *mariner* insertion are certain to harbor essential genes¹⁸⁵. A similar approach for insertional mutagenesis *in vitro* has also been proposed with the retrotransposase Ty1 of *S. cerevisiae*⁶⁰, although its use is far less spread.

A final improvement regarding the use of mini-transposons worth mentioning here is the development of various procedures for rapid sequencing of the DNA sequences adjacent to the site of the insertions. This is achieved by PCR-amplification of transposon-chromosomal DNA junction sequences, followed by determination of the transposon insertion site by nucleotide sequencing^{103, 114, 115}. The amplification of junction fragments consists of two PCR reactions. In the first reaction, chromosomal DNA is amplified using two primers. One of these primers (primer 1) is designed to hybridize to a defined transposon sequence and the other (primer 2) is actually a degenerate mixture of oligonucleotides designed to hybridize at many sites in the genome. Primer 2 also introduces a unique sequence tag at its 5' end. In the second PCR reaction, the amplicons of the first step are amplified using a different, nested transposon-specific primer (primer 3) and a primer (primer 4) hybridizing to the unique sequence tag introduced by primer 2. This procedure works best with strains of known genomic sequence, but can also be applied to any other strain. Furthermore, the procedure simply requires the DNA to be released into the supernatant of a single boiled colony of the mutant(s) and is thus suitable for high-throughput analysis¹¹⁵.

8. POST-GENOMIC GENETIC TOOLS

Whole genome sequences have shown that bacteria possess a significant number of genes that have no known function. It is probable that many of these

are required for survival in environments other than the agar plate or similar laboratory conditions, thereby demanding *in vivo* selection strategies to reveal genes that are active in complex natural environments. In other cases, the issue is the identification of genes that are present in strains other than those which have been entirely sequenced. A few procedures developed to address some of these issues are briefly outlined in the following paragraphs.

First, recent years have witnessed the emergence of a whole collection of genetic strategies generally known as *in vivo* expression technologies (IVET). This topic is the subject of Chapter 11 of this volume and is not discussed in detail here. In general, any IVET strategy relies on the random cloning of promoter libraries for driving expression of an essential or conditionally essential gene¹²⁶. Such libraries can be prepared in plasmids and then recombined into the chromosome. When populations of cells bearing promoter fusions of this kind are placed under restrictive *in vivo* conditions, only those that express the essential genes do survive. These promoter-trapping procedures can be repeated several times until a small collection of representative promoters is enriched. Host/gene pairs for IVET schemes include adenine-requiring auxotrophic mutants of *P. aeruginosa* and the *purEK* operon^{173, 174}, *P. putida pyrB* mutants and the *pyrB* gene (which encodes aspartate transcarbamoylase, an enzyme used for pyrimidine biosynthesis), or diaminopimelic auxotrophs in combination with the corresponding synthetase gene¹²⁶. These procedures have been instrumental in identifying genes involved in a large variety of traits necessary for survival and proliferation in given environments^{15, 95, 121, 124–126, 162, 173, 174}.

A second procedure to identify genes that are exclusively expressed under defined *in vivo* conditions is the one known as *signature tagged mutagenesis* (STM). In its simpler version⁶⁹, the procedure involves the separate mutagenesis of the strain under scrutiny with pools of transposons each labeled with a predetermined oligonucleotide tag. After passing the whole population of mutants through restrictive *in vivo* conditions, those tags that are lost after recovery of the cells are candidates containing transposons inserted in genes important for thriving in the specific test site. To this end, the tags from a mixed population of bacterial mutants representing the inoculum and bacteria recovered from the inoculated niche are detected by amplification, radiolabeling, and hybridization analysis. Many variations of the STM technique are now possible and the method can be applied to strains (e.g., pathogens) that have an STM-scorable phenotype in a host system. As a genome-scanning tool, STM will yield information about genes with unknown functions as well as information crucial for understanding microbial pathogenesis and survival in various environmental niches⁹⁷. Other, less used but equally valuable transposon-based approaches to identify the functions of orphan genes include the *essential gene test* (EGT, a transposon-based

technique that can rapidly identify a nucleotide sequence from a database as essential or dispensable)⁹⁸, and the genomic analysis and mapping by in vitro transposition (GAMBIT)¹. Some of these procedures were not initially developed for *Pseudomonas*, but can be applied to this and other genera of Gram-negative bacteria.

A third group of post-genomic tools involve genetic strategies to identify DNA sequences not shared by two or more strains. The concept in this case is the use of a variety of subtractive techniques to recover genes present in one isolate but not the other, such as those found on pathogenicity islands. The procedure of choice here is known as representational difference analysis or RDA²¹. This method, which originates in cancer research, involves the subtractive hybridization and kinetic enrichment that has been used previously to recover differences between two complex genomes, including identifying the genome of human herpesvirus 8 in the tissue of patients with Kaposi's sarcoma. RDA has also been modified to utilize cDNA as the starting material, thereby allowing analysis of differential gene expression. More recently, RDA was adapted for use in detecting and cloning genomic differences between two closely related bacterial species or isolates of the same species. The result of this procedure is the sequential enrichment of PCR products resulting from DNA sequences not shared by any two strains. The method has been very successfully employed to directly identify genomic differences between *P. aeruginosa* strains PA14 and PAO1, and undoubtedly has a considerable potential in many other instances²¹.

9. OUTLOOK

Even though the arsenal of genetic tools for genetic analyses of pseudomonads has steadily improved over the last 10 years, it still lags behind to that available for *E. coli* and there is still room for considerable improvement in many of the areas that have been addressed in this chapter. More efficient genetic tools are needed to complement other rapid analytical tools, including microarrays and proteomic technologies. The following is merely a short list of desirable improvements.

Many of the broad-host-range vectors available for *pseudomonads* do not allow for visible screening of recombinants and only the β -galactosidase based blue/white screening has been employed on a very limited basis. Other screenable markers have not yet been applied in pseudomonads. One prospect is the *celA*-encoded endoglucanase A (cellulase) marker from *Clostridium thermocellum*^{77, 119}. *E. coli* strains containing plasmids that express a plasmid-encoded *celA* gene form clear halos on cellulose-containing media. Interruption of the *celA* gene by cloning DNA fragments into a MCS located

within this gene leads to loss of this halo. Provided that the respective pseudomonads do not express an endogenous cellulase, the *celA* gene could easily be incorporated into diverse broad-host-range vectors and provide a cheap alternative visual selection to the traditional XGal based blue/white screening.

In many instances, positive selection may be a preferred alternative to screening based on loss of a phenotype. Positive *E. coli* selection vectors have been described that are based on *ccdB*. This gene is derived from the F plasmid and encodes a bacterial cytotoxin targeting bacterial DNA gyrase^{8, 9, 11}. CcdB traps the tetrameric A₂B₂ DNA gyrase in a complex with the gyrase subunit covalently linked to the cleaved DNA. CcdB resistant mutants contain defined *gyrA* mutations¹⁰ and can therefore be used to stably propagate cloning vectors containing the *ccdB* gene. When *ccdB* is inactivated by cloning DNA fragments into a multiple cloning site located in its promoter-proximal end, stable transformants of *gyrA*⁺ host strains can be obtained, whereas vectors without inserts will kill the same hosts. Since evidence was obtained that CcdB is active against *P. aeruginosa* gyrase (cited in ref. [9]), it is conceivable that the same is true for other pseudomonads. Thus, broad-host-range vectors could be derived from available *E. coli* vectors and be used in conjunction with appropriately engineered *gyrA* mutants.

Direct cloning in *Pseudomonas* is still hampered by lack of appropriately engineered host strains. Such strains should be easy to transform, allow screening or positive selection of recombinants, stably maintain recombinant DNA, and allow isolation of high-quality and intact plasmid DNA. With the advent of genome sequencing the genes responsible for these traits (e.g., *endA*, *recA*, and others) can be identified, and the genetic technologies required to manipulate and engineer them are available. Such genetically engineered host strains should preferably be unmarked so that plasmid antibiotic resistance markers are not compromised in these hosts. Suitable broad-host-range technologies to achieve this already exist, for example, the FLP-*FRT* system⁷¹ or the ParA-*res* system⁹¹.

Bacterial genomes contain many genes of unknown functions and the genomes of pseudomonads are no exception. To facilitate genetic analyses of the many genes encoded by these genomes, rapid mutagenesis procedures are required. Although the *sacB*-based gene replacement procedure described in this chapter is being successfully used in different pseudomonads, it suffers from problems that are inherent to this technology. A recently developed simple one-step gene replacement procedure for *E. coli* that bypasses the need for cloning and suicide plasmid delivery may help accelerate the mutagenesis process in pseudomonads³³. This method employs short PCR *homing* primers that provide the homology to the targeted genes. The antibiotic resistance cassette intended for insertional inactivation of the gene of interest is PCR-amplified from a

plasmid template using primers that are homologous to plasmid sequences and also to target DNA sequences (*homing* primers). When the PCR fragments is used to transform a host strain expressing the γ /*red* system of phage λ recombination between the short (36–50 bp) homing primers and their homologous chromosomal DNA target sequences is mediated by the λ Red recombinase, encoded by the β , γ , and *exo* genes. Although initial attempts at adapting the procedure to strains of different *Pseudomonas* species failed in several laboratories, the λ RED recombinase system (or an equivalent procedure) still holds promise as a future genetic tool for pseudomonads.

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IN VIVO GENE EXPRESSION: THE IVET SYSTEM

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1. INTRODUCTION

Bacteria of the genus *Pseudomonas* have been found in quite diverse aerobic environments, their metabolic versatility and capacity to adapt to changing conditions being a major reason for their ubiquity. A classical approach in studies carried out with pseudomonads and other bacteria, has been the analysis of a specific phenotype of interest and the subsequent identification of the gene/s responsible for such character, either by inactivation of a suspect gene or by random mutagenesis. Expression patterns of many genes have been investigated under laboratory conditions, and in vivo gene activity has in some cases been documented. However, as more complex inquiries about the biology of *Pseudomonas* in specific environments have been undertaken, the limitations of this genetic approach have become apparent. The interactions of bacteria with their environment, or with other organisms, are determined by a number of different factors—not only the bacterium's particular genetic stock, but also those of surrounding bacteria, or of the organisms they interact with, as well as physicochemical conditions. Thus, the ecological performance of a bacterium is the result of a combination of intrinsic and external elements, and it is often difficult

to assess the role of one particular gene product simply by its inactivation. This is the case in human pathogenesis, plant virulence, and plant protection against infection by pathogens—all conditions involving *Pseudomonas* sp. In such situations, confirmation of the role of a certain gene in bacterial fitness has quite often required competition studies between wild-type and mutant strains, indicating the existence of genes whose contribution to the adaptation of some bacteria to their natural habitats depends upon their bio-environment. Often these genes have been found to be unimportant for growth in the laboratory.

Identification of genes by in vivo expression technology (IVET) is based on their contribution to a specific phenotype, as opposed to identification strategies based on function loss. This is of major ecological significance, since it implies selection in competition. In this chapter we will describe this technology, its variants, and their uses to advance our knowledge of the biology of *Pseudomonas*.

2. DESCRIPTION OF THE IVET SYSTEM

The term IVET was coined 10 years ago by Mahan and coworkers¹⁴ to refer to a positive selection system for genes that were induced when the pathogen *Salmonella typhimurium* infected its host. Since then, the IVET system has been successfully used in the identification of bacterial virulence factors. The aim of the IVET system, which is the selection of in vivo-induced genes, had already arisen in previous work carried out with *Xanthomonas*¹⁶. On that occasion bacterial promoters were selected for their ability to command the expression of a promoterless *cat* (chloramphenicol acetyl transferase) gene during infection of turnip seedlings. Such a strategy, based on antibiotic treatment for in vivo selection can be applied under some experimental situations, for example, to study pathogens that adopt an intracellular lifestyle during infection. This strategy works because chloramphenicol can penetrate mammalian cells¹⁵. However, selection based on antibiotics is not always feasible in vivo and alternative methods of selection have been developed. These methods are based on growth requirements that bacteria are unable to synthesize, and which are not available in the environment where selection takes place.

2.1. The IVET Strategy

The IVET approach consists of a gene expression reporter cassette that permits the generation of transcriptional fusions, which can then be selected on the basis of their in vivo induction. The process used to identify functions of ecological relevance in a bacterial strain by a standard IVET system is summarized in Figure 1.

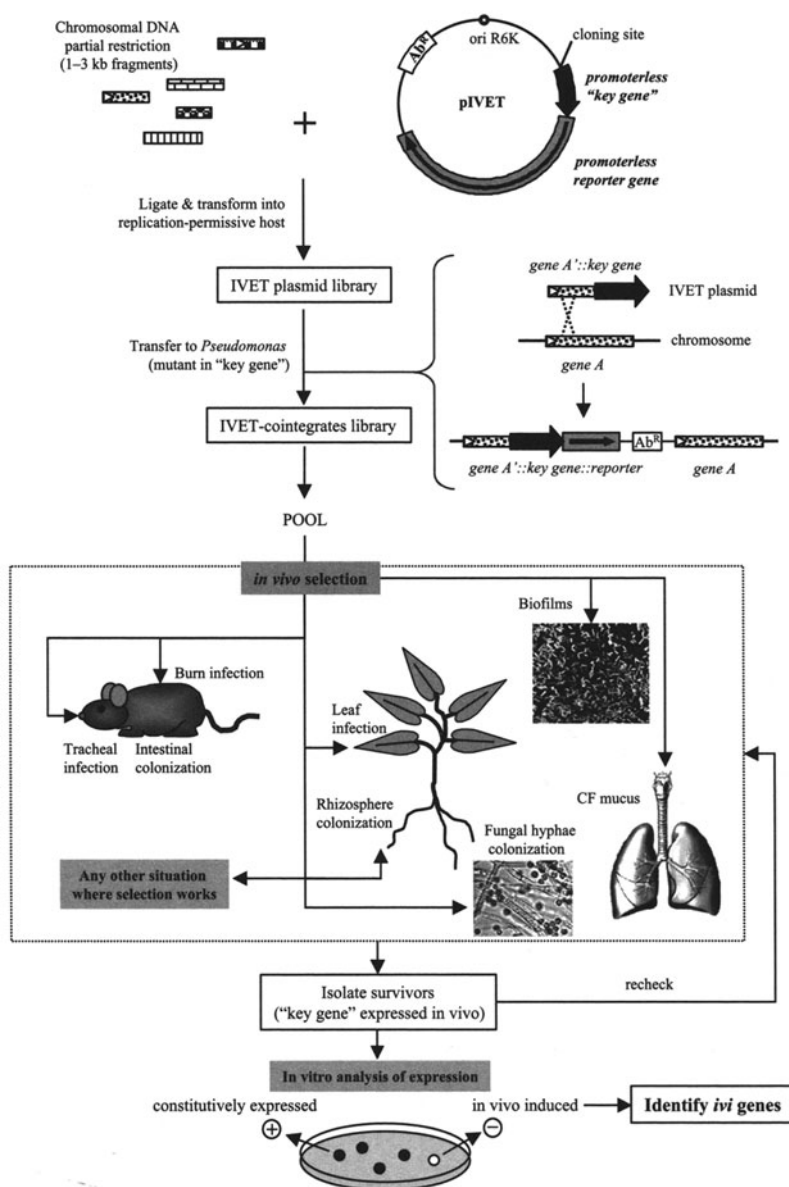


Figure 1. Isolation of in vivo-induced fusions. The first step consists of the construction of a gene bank of the bacterial genome of interest in the IVET plasmid. The resulting IVET library is transferred *en masse* to a mutant *Pseudomonas* strain bearing a deletion in a locus that is essential under the screening conditions ("key gene"). The chromosomal IVET-cointegrates library, which is generated by homologous recombination, is checked by *in vivo* selection. The reporter gene in the IVET plasmid confers the possibility to discriminate between constitutively and specifically induced genes.

The first element required is a null mutant in a “key gene” whose product is essential for bacterial survival under the particular environmental conditions being studied; this is the case of mutations that cause auxotrophy and/or lethality in the absence of specific growth factors. The second requisite is a promoter probe suicide vector, which carries the “key gene” deprived of its promoter (IVET plasmid). This vector is used to construct a genebank of the bacterial genome, such that in those clones whose inserts exhibit promoter activity, transcription of the essential gene will take place. The resulting IVET library is transferred *en masse* to a mutant strain bearing a deletion in the essential locus. The outcome is a genebank of exconjugants with the chimeric IVET plasmids cointegrated at the loci corresponding to the plasmid inserts, by homologous recombination (hence the use of null mutants to avoid undesired integration of the IVET plasmid at the “key gene”). Because IVET is a nondisruptive technique, merodiploids, in contrast to transposon insertions that disrupt target genes, present mostly intact copies of the targeted loci whose promoters, in their native chromosomal context, are responsible for the expression of the essential gene. The collection of exconjugants can then be tested in the desired environment. Activation of the *in vivo*-induced (*ivi*) fusions leads to complementation of the mutant phenotype, which results in survival even in the absence of the growth requirements mentioned above. As a result of the *in vivo* screening of the chromosomal cointegrate library for the positive selection of clones, which are able to withstand a specific situation, a battery of active genes is identified. A promoterless reporter gene downstream the “key gene” is usually present in IVET plasmids as an *in vitro* tool, useful to differentiate between constitutive and specifically *in vivo*-induced promoters. The chromosomal context eliminates the high copy number effect, often a problem in fusions contained in plasmids. Genes of special ecological significance are on *in vivo* and off *in vitro*.

2.2. Methods for the Analysis of *In vivo*-Induced Fusions

IVET-generated gene fusions are maintained integrated in the chromosome of the host bacterium. Because efficient transductional methods are not available for *Pseudomonas*, alternative techniques to analyze the *in vivo*-induced fusions have been developed.

A genetic approach for the recovery of chromosomally-integrated fusion plasmids from pseudomonads is the conjugative cloning system reported by Rainey and coworkers²². In a triparental conjugation consisting of a recipient strain, which permits replication of the IVET plasmid, and a helper strain supplying *mob* functions *in trans*, recovery of the integrated fusion plasmids from the chromosome of the donors can be achieved by homologous recombination. Conjugative cloning is a fast and simple alternative to the more tedious

process of isolating chromosomal DNA, cutting, religating, and transforming the fusion-containing IVET plasmids.

Target genes can be identified by direct sequencing of the fusion from the chromosome, after PCR amplification with arbitrary primers^{3,6}. Arbitrary PCR for the analysis of fusions is useful for chromosomes whose sequences are already available in the databases.

Analysis of the expression pattern of promoters identified with the IVET approach takes advantage of the reporter gene included in the IVET vector. The quantification of in vivo gene expression requires highly sensitive methods of detection since the number of cells in the assay can be very low.

2.3. Variants of IVET

Plasmids based on different selectable markers and reporter genes have been developed for IVET (IVET plasmids), and their use in *Pseudomonas* studies has been reported. Most of these are listed in Table 1.

A number of biosynthetic loci exist whose inactivation renders the bacterium unable to grow under specific situations in vivo. These loci are therefore candidates for use in IVET systems. They include genes responsible for the synthesis of purines and pyrimidines (*purA*, *purEK*, and *pyrBC*)^{13, 14, 26, 31}; pantothenate, a B-group vitamin essential for the biosynthesis of CoA (*panB*)²¹; diaminopimelic acid (DAP), a component of the cell wall peptidoglycan of gram-negative bacteria (*asd*)¹¹. The product of the *asd* gene, in addition to being an essential enzyme in DAP biosynthesis, is part of the biosynthetic pathway for Lys (see details in Chapter 12, vol. III), Met, and Thr. These metabolites are not available for bacteria in certain environments, therefore the genes responsible for their biosynthesis are essential genes of interest for IVET systems. As reporter genes, *lacZY* for β -galactosidase and *uidA* for β -glucuronidase have been incorporated into IVET plasmids.

2.4. Applications of IVET as a Tool in Biotechnology

The reporter genes incorporated in the IVET cassette to analyze *ivi* fusions in vitro are potentially useful to isolate repressors of the in vivo-induced genes, in the way that they can be introduced into a specific mutant (or a pool of random mutants). Mutant hosts bearing *ivi* fusions that are induced in vitro, which otherwise were silent in the wild type as a host, are candidates to present mutations in repressors of the *ivi* genes¹.

Different alternatives to the basic IVET have been developed. A promoter-trap method to identify infection-induced genes, termed RIVET, was designed to elucidate when and where specific genes are expressed. This method uses, as transcriptional reporter, a gene encoding a site-specific DNA recombinase

Table 1. IVET selections used to identify *Pseudomonas* in vivo-induced genes.

<i>Pseudomonas</i> sp.	In vivo screen	Selection ^a (antibiotic resistance)	Reporter gene ^b	Num. genes identified ^c	References
<i>P. aeruginosa</i>	Respiratory mucus from CF patients ^d	<i>purEK</i> (Tc, Ap)	—	3	[30]
	Systemic infection in neutropenic mouse	<i>purEK</i> (Tc, Ap)	—	22 ^e	[31]
	Burned mouse infection	<i>purEK</i> (Tc, Ap)	—	4	[8]
	Systemic infection in mouse	<i>purA</i> (Cb)	—	8 ^f	[10]
	Chronic lung infection in rat	<i>purA</i> (Cb)	<i>lacZY</i>	6	[10]
	Mature biofilm	<i>purEK</i> (Tc, Ap)	—	5	[7]
<i>P. fluorescens</i>	Root colonization of sugar beet seedlings	<i>dapB</i> (Tc)	<i>lacZY</i>	20 ^g	[21]
<i>P. putida</i>	Colonization of plant-pathogenic fungus	<i>pyrBC</i> (Tc)	<i>lacZ</i>	5	[13]
<i>P. syringae</i>	Root colonization of maize seedlings	<i>asd</i> (Km)	<i>lacZY</i>	25	[24]
	Infection of <i>Arabidopsis thaliana</i>	<i>hrcC</i> (Km)	<i>uidA</i>	58 ^h	[2]

^aBiosynthetic locus for purines (*purEK*, *purA*); pyrimidines (*pyrBC*); pantothenate (*dapB*); diaminopimelic acid (DAP) (*asd*). *hrcC* encodes an outer-membrane type III secretion protein essential for plant pathogenesis. *asd* encodes aspartate β-semialdehyde dehydrogenase; a mutant in *asd* causes auxotrophy for amino acids Lys, Met and Thr in addition to DAP.

^b*lacZ*, β-galactosidase; *lacY*, lactose permease; *uidA*, β-glucuronidase.

^cNumber of genes identified in the screen with the IVET system.

^dCystic fibrosis.

^{e,h,g}List of genes compiled in tables are shown in the references cited.

that catalyzes excision of a selectable substrate gene cassette (for example antibiotic resistance) from the bacterial genome^{1, 4, 12}. This system makes it possible to select fusions that were induced in vivo at some point during screening, even if it was for a short time, and requires replica plating tests in order to detect those which underwent recombination and therefore lost the selectable marker.

The IVET technology can be exploited for biotechnological purposes related with medical applications. Vaccines against pathogens can be generated by identifying surface proteins adsorbed to the patients' serum. This approach for studying microbial pathogenesis is termed IVIAT (in vivo-induced antigen technology). This method is potentially useful to identify genes expressed during human infection, since the system uses sera from patients to probe for genes specifically expressed in vivo⁹.

Because the IVET system assists the identification of in vivo-induced genes, it may increase our knowledge of the causes for ecological performance. In this sense, the system provides an important tool for the design of biocontrol strains and strains for industrial fermentations²³.

3. APPLICATIONS OF IVET IN *PSEUDOMONAS*

The IVET system is a valuable technology to identify genes that are expressed in a wide variety of situations in vivo. In Table 1, specific selections and screens used to identify *Pseudomonas* in vivo-induced genes are listed. In this section we will review the most significant uses and results obtained in different *Pseudomonas* species.

3.1. *Pseudomonas aeruginosa*

The IVET system has been used to screen for *P. aeruginosa* candidate genes that affect virulence in vivo. Different models have been utilized as hosts for infection: Intraperitoneally injected mice (septicemia model)^{10, 31}; burned mouse infection model⁸; and intratracheally treated rats (chronic lung infection model)¹⁰. Another approach has been the selection of promoters inducible by respiratory mucus from patients with cystic fibrosis (CF)³⁰, although mucus from non-CF individuals was also capable of inducing the expression of the same genes. It should not be overlooked that *ivi* genes are selected on the basis of their expression during infection, and not for encoding virulence determinants, as pointed out by Rahme and coworkers²⁰. In the models noted above, the IVET system was based on the biosynthesis of purines, since purine auxotroph derivatives of *P. aeruginosa* were avirulent³¹. Two independent chromosomal cointegrated libraries were used in these studies,

one generated from the clinical isolate PAK³¹ and the other from PAO909¹⁰. Two out of eight *ivi* fusions were genes exclusively recovered from mouse, whereas the other six were common to both mouse and rat models¹⁰. The identification of the same loci by independent methods of selection may reflect their general role in pathogenesis (Table 2). This is the case for genes related to iron acquisition, such as *fptA*, encoding a pyochelin receptor, which is induced by mucus from CF patients and in neutropenic mice^{30, 31}. The *fptA* gene was confirmed to be repressed in abundance of iron in vitro³⁰. Iron deficiency seems to be a host defense mechanism against infection. A transcriptional regulator homologous to Fur (*np20*) was also identified as being active in neutropenic mice and in the presence of mucus^{30, 31}.

Other promoters recovered from mouse and rat models command the expression of proteins homologous to FtsY, which participates in membrane biogenesis and is involved in transport and secretion. Some promoters have been recovered exclusively from one experimental model, like *migA*, which was isolated as being inducible by mucus from CF patients. This gene encodes a homolog of glycosyltransferases with a role in biosynthesis of LPS and/or EPS³⁰.

A total of 22 loci were identified as being inducible during infection of neutropenic mice (Table 1). From their sequence it was inferred that they were involved in gene regulation, signal transduction, and amino acid biosynthesis. An average of 30 percent of the identified loci were of unknown function³¹.

The IVET system developed by Wang and coworkers was applied to the burned mouse infection model, and four specifically induced *ivi* fusions were identified⁸. The identification of a gene for a superoxide response regulator, SoxR, which functions as a bacterial defense system against oxidative stress, is evidence that oxidative stress is an important feature of burn wounds. Also identified were *glcG*, encoding a conserved hypothetical protein of unknown function, which is part of the glycolate oxidase cluster *glcCDEF*; an antisense for a two-component response regulator; and a gene of unknown function was also found.

The chromosomal cointegrate IVET library published by Wang and coworkers has been recently used to identify *P. aeruginosa* genes expressed in biofilm, a methodology which the authors coined as IBET (in biofilm expression technology)⁷. Five genes not overlapping with previously known biofilm formation loci were identified, which is in agreement with the suspected existence of multiple pathways to acquire a sessile lifestyle¹⁷. One of the IBET fusions revealed a homologue of *E. coli ubiB*, which is involved in ubiquinone biosynthesis. An interesting IBET gene encodes a regulator of the AraC/XylS family, similar to the *Streptomyces griseus* developmental regulator *adpA*. Inactivation of *ubiB* is lethal; however, inactivation of the *adpA* homologue and two other loci, a putative porin and a putative alcohol dehydrogenase,

Table 2. Functions of the *P. aeruginosa* in vivo-induced genes identified during mammal infection^a.

In vivo screen	Function and/or role	IVET fusion and/or gene	Locus ^b
Mouse			
Systemic infection	Iron scavenging		
	Pyochelin mediated iron acquisition	<i>np22 (fptA)</i>	PA4221
	Transcriptional regulator (Fur family)	<i>np20</i>	PA5499
	Chemotaxis regulator	<i>np9 (cheY)</i>	PA1456
	Amino acid biosynthesis		
	Threonine dehydratase (Ile)	<i>np16 (ilvA)</i>	P04968
	Imidazole glycerol-phosphate dehydratase (His)	<i>np18 (hisB)</i>	PA5143
	Cobalamin biosynthesis	<i>np4 (cobI)</i>	PA2904
	Serine/threonine protein kinase	<i>np6</i>	P33973
	RNA helicase (ribosome assembly)	<i>np13 (dbpA)</i>	PA0455
	Resolvase (phage integrase family)	<i>np19</i>	P06615
	Transcriptional activator (LysR family)	<i>np10</i>	P42427
	Docking protein (transport and secretion)	<i>ivi134-21 (ftsY)</i>	PA0373
	Conflicting		
	Probable nonribosomal peptide synthetase ^d	<i>ivi131-17 (pvdD)</i>	AF214674
	Adhesion, colonization ^d	<i>ivi131-19 (hag2)</i>	AF214675
	Others (up to 16)		
	Generic predictions		
	No predictions		
	No similarity in the minus strand		
Burn infection	Glycolate utilization	<i>sf7 (glcG)</i>	PA5352
	Response to oxidative stress	<i>sf21 (soxR)</i>	PA2273
	Antisense RNA for a transcriptional regulator	<i>sf17</i>	L05176
	Unknown	<i>sf2</i>	
Respiratory mucus ^c	Iron acquisition		
	Pyochelin mediated iron acquisition	<i>fptA</i>	PA4221
	Transcriptional regulator (Fur family)	<i>np20</i>	PA5499
	Glycosyltransferase for biosynthesis of LPS or EPS	<i>migA</i>	PA0705

^aHighlighted in gray, genes whose role in infection was confirmed in studies with mutants generated in these loci.

^bLocus of *P. aeruginosa* PAO1 or best hit with databases. Except for the so-called *ivi* and *np20* fusions, there is no sequence information for IVET fusions. When possible, *P. aeruginosa* loci were deduced from similarity data given in the original studies. Where the *P. aeruginosa* locus could not be inferred, the best hit is given, as provided in ref. 31 for the *np* and ref. 8 for the *sf* fusion.

^cMucus was obtained from patients with cystic fibrosis.

^dThe best hit and hence the function suggested in the original study¹⁰ does not correspond to the updated annotation of the *P. aeruginosa* genome sequence (March, 2003). Original entries for the fusions are given in these cases.

affected biofilm formation, development and fitness, but no effect was observed on planktonic growth⁷.

3.2. *Pseudomonas syringae*

A modified IVET approach has been used to identify promoters from the plant pathogen *P. syringae* pv. tomato that can direct in planta expression (IPET) of a promoterless *hrcC* gene². Thus, instead of a biosynthetic gene, the IPET system is based on an essential gene for plant pathogenesis²⁷. Since an *hrcC* mutant is defective for type III secretion and hence unable to grow in plant tissue, the strategy involves selecting promoters capable of restoring growth of the *hrcC* mutant in plant tissue. The model used was infection of *Arabidopsis thaliana*, and the loci identified with this system were named in planta-expressed (*ipx*) (Table 3). More than 50 independent *ipx* fusions were identified with this system, 22 of which presented the consensus “*hrp* box” sequence motif and hence were candidates to belong to the pathogenesis-related regulon governed by the alternative RNA polymerase sigma factor HrpL^{28, 29}. All but one exhibited HrpL-dependent expression as tested in *E. coli* expression assays of the IPET plasmids. This group included fusions to *hrp/hrc* genes (encoding a specialized type III protein secretion apparatus), *avr* (avirulence) and *vir* (virulence) fusions, and fusions to genes in the conserved effector locus (CEL) flanking the *hrp/hrc* cluster⁵. Of the *ipx* fusions identified as being HrpL-dependent, one was to a gene encoding a putative pectin lyase, which may be involved in degrading plant cell wall, some exhibited no similarity to entries in the databases, some showed similarities to hypothetical proteins, and one fusion identified a putative Rhs (rearrangement hot-spots) family protein. These fusions may encode novel virulence factors that have yet to be characterized.

The existence of other regulatory factors was suggested based on the evidence that some *ipx* fusions exhibited induction after infection of plant tissue despite the observed lack of HrpL dependence in the *E. coli* expression assay. Thirteen of the *ipx* fusions were to genes related with biosynthesis of the phytotoxin coronatine, a process that seems to be coordinated with the expression of the *hrp/hrc* system¹⁸. In addition to the known virulence genes and novel potential virulence genes, the IVET screen identified metabolic and biosynthetic genes with unknown function, presumably important for the adaptation of bacteria to grow on plant tissue. These unidentified genes are likely involved in amino acid synthesis and in maintenance and modification of the bacterial cell envelope.

One out of four mutants generated by the inactivation of genes identified as *ipx* fusions, which mapped to an ORF within the CEL, exhibited a significant

Table 3. Function of the *P. syringae ipx* genes identified during plant infection^a.

Function and/or role	Gene and/or IPEX fusion	Locus ^b
Pathogenesis (known or predicted)		
Coronatine biosynthesis		
Coronafacate ligase	<i>cfl</i>	PSPTO4680
CFA-acyl carrier protein	<i>cfa1</i>	PSPTO4681
Coronafacic acid synthetase component	<i>cfa5</i>	PSPTO4685
Type I polyketide synthase	<i>cfa6</i>	PSPTO4686
Type II polyketide synthase	<i>cfa7</i>	PSPTO4687
Crotonyl-CoA reductase Cfa8	<i>cfa8</i>	PSPTO4689
CFA synthetase thioesterase component	<i>cfa9</i>	PSPTO4690
Nonribosomal peptide synthetase	<i>ipx29</i>	PSPTO4699
Alginate biosynthesis	<i>algA</i>	PSPTO1232
Membrane-bound lytic murein transglycosylase D	CEL ORF1	PSPTO1378
Stress response		
Copper translocating P-type ATPase (copper resistance)	<i>ipx33</i>	PSPTO0750
Catalase (resistance to oxidative stress)	<i>katB</i>	PSPTO3582
Type III helper protein pectin lyase	<i>hopPmaH(Pto)</i>	PSPTO4101
Type III secretion system and effectors proteins		
HrpJ	<i>hrpJ</i>	PSPTO1403
HrpG	<i>hrpG</i>	PSPTO1388
HrcQb	<i>hrcQb</i>	PSPTO1396
ATP-dependent helicase HrpA	<i>hrpA</i>	PSPTO4095
Type III helper protein HrpK(Pto)	<i>hrpK(Pto)</i>	PSPTO1405
Effector HopPtoM CEL ORF3	<i>ipx12</i>	PSPTO1375
Effector HopPtoN CEL ORF7	<i>ipx13</i>	PSPTO1370
Conserved effector locus protein	<i>ipx15</i>	PSPTO1369
Effector HopPtoA2	<i>ipx32</i>	PSPTO4718
Avirulence protein AvrPtoB	<i>avrPtoB (ipx7)</i>	PSPTO3087
Avirulence protein AvrPpiB1(Pto)	<i>avrPpiB1(Pto)(ipx8)</i>	PSPTO1022
Candidate effector Hop protein	<i>ipx39</i>	PSPTO0836
Others		
Cell wall recycling (muropeptide transporter)	<i>ampG</i>	PSPTO4383
Long-chain fatty acid CoA ligase	<i>fadD-1</i>	PSPTO4097
Nutrient acquisition		
Pyruvate dehydrogenase	<i>aceE-2</i>	PSPTO5005
Acetolactate synthase	<i>ilvB</i>	PSPTO0981
Gly cleavage complex protein H	<i>gcvH-1</i>	PSPTO0317
DAP decarboxylase (Lys biosynthesis)	<i>lsyA-1</i>	PSPTO0209
Thiamine biosynthesis lipoprotein	<i>ipx45</i>	PSPTO2105
Amino acid transport and metabolism	<i>ipx35</i>	PSPTO0939
Transposase	<i>ipx50</i>	PSPTO1063
ATP binding component of branched-chain aa ABC-transporter	<i>ipx46</i>	PSPTO4075 ^c
Carboxylase (nikkomycin biosynthesis domain protein)	<i>ipx47</i>	PSPTO0874

Table 3. Continued

Function and/or role	Gene and/or IPEX fusion	Locus ^b
Two-component sensor response regulator (LuxR family)	<i>ipx49</i>	PSPTO0897
Sensor histidine kinase	<i>ipx50</i>	PSPTO4291
Rhs family protein	<i>ipx52</i>	PSPTO0373
Unknown (up to 17)		

^aHighlighted in dark gray, a gene whose role in plant infection was confirmed in studies with a mutant in this locus². In pale gray, those genes whose role in infection was ruled out².

^bLoci of *P. syringae* pv. tomato str. DC3000 was deduced from the *ipex* fusions (supplementary material to ref. 2, <http://www.blackwell-science.com/products/journals/suppmat/mole/mole2877/mmi2877sm.htm>).

^cLocus as annotated in the release of the complete genome (March, 2003) does not retrieve any sequence; a productive entry is AAF71487.

alteration in pathogenesis (Table 3). The finding that the other inactivations, including that of pectin lyase, did not exert any effect upon virulence might be explained as a consequence of redundancy in virulence factors that are unlikely to be identified by screening mutants according to loss of function.

3.3. *Pseudomonas fluorescens*

The IVET strategy has been used in *P. fluorescens* SWB25 to identify genes of ecological importance for the colonization of sugar beet seedling roots by this plant-growth-promoting rhizobacterium (*rhizosphere-induced genes, rhi*)²¹. A mutant auxotrophic for pantothenate was used as a host for the IVET-cointegrates genebank. Initially, more than 20 *rhi* genes were identified as involved in nutrient acquisition, stress response or secretion; no function could be assigned to 40 percent of them (Table 4). Two of the *rhi* fusions with a likely role in nutrient acquisition were to genes involved in amino acid uptake. Another fusion was associated with a gene that probably encodes an oxidoreductase involved in the utilization of complex nitrogen compounds. One fusion was to a gene with a role in xylose metabolism, a sugar present in root exudates. Two stress response elements were also identified, a two-component system regulator involved in metal resistance and an AhpC-TSA family protein with a role in protein turnover. Among the secretion genes identified, one showed homology to type III secretion system genes, a finding that may indicate the existence of a specific, intimate interaction between plants and beneficial bacteria¹⁹. More than a hundred loci have been identified with this system of in vivo screening so far. A comprehensive list of these genes is available at the website (www.plants.ox.ac.uk/sbw25/).

Table 4. Function of the *Pseudomonas fluorescens rhi* genes identified during sugar beet root colonization.

Function and/or role	<i>rhi</i> -fusions and/or gene	Locus ^a
Nutrient acquisition		
Xylose metabolism	<i>rhi-17 (xylA)</i>	PSPTO3002
Probable oxidoreductase ^b	<i>rhi-4</i>	Avin0590
Branched-chain amino acid transport permease	<i>rhi-10</i>	Psyr3724
Putative histidine transport protein	<i>rhi-14 (hutT)</i>	PP5031
Secretion		
Trigger factor (protein export and cell division)	<i>rhi-15</i>	PP2299
Outer membrane type III secretion porin ^c	<i>rhi-18(hrcC)</i>	PSPTO1389
Cation efflux or multidrug resistance protein	<i>rhi-8 (ragC)</i>	Blr2934
Stress response		
Sensor histidine kinase (metal resistance) ^b	<i>rhi-3</i>	PP2157
Thiol-specific antioxidant protein ^d	<i>rhi-12 (lsfA)</i>	PP0235
Unknown		
Probable transcriptional regulator ^b	<i>rhi-5</i>	PA2825
Putative transcriptional regulator ^b	<i>rhi-1</i>	VC1772
Probable sulfatase ^d	<i>rhi-7</i>	Pflu0951
Leucine-rich repeat domain protein	<i>rhi-9</i>	PSPTO1492
Hypothetical protein	<i>rhi-11</i>	PP2660
Sensor histidine kinase ^b	<i>rhi-19</i>	Pflu0552

^aBest hit to predicted proteins according to the *rhi* sequence entries available at http://enterprise.molbiol.ox.ac.uk/~aspier/PfSBW25_Encyclopedia/EPhtml. No entries were available for the *rhi*-fusions in the databases as such and therefore only those that coincide both in ref. 21 and in the above website were included.

^bDifferences with respect to the original report²¹ as a consequence of databases update.

^cThe role of the type III gene cluster in root colonization and competitive colonization was analyzed and no significant effect was observed¹⁹.

^dFusions to the minus strand as inferred from *rhi*-fusion sequences available in the website.

3.4. *Pseudomonas putida*

Studies of gene expression in *P. putida* KT2440 during root colonization using maize as a model plant are being carried out at present, and more than 20 rhizosphere-activated promoters (*rap*) have been identified²⁴. An *asd* null mutant was used, as it is unable to survive in the rhizosphere due to its dependence on DAP acid, Lys, Thr and Met²⁵. Among the *rap* fusions, work so far has identified genes encoding transcription factors, DNA-rearrangement, secretion apparatus proteins, amino acid transporters, and metabolic (anabolic and catabolic) enzymes, in addition to conserved hypothetical proteins with unknown functions. Of special note is the lack of overlap between the results obtained with *P. fluorescens* and *P. putida*. This emphasizes that either none of the studies are saturated and/or that plant interactions with each bacterial species are

unique and multiple studies will be required to gain a broad understanding of how bacteria adapt to the rhizosphere.

The IVET strategy has been used to investigate colonization of the plant-pathogenic fungus *Phytophthora parasitica* by *P. putida* 06909 based on the absence of pyrimidine in the fungal environment¹³. The results thus far show that 19 *ivi* clones out of 30 identified in the screen were induced at the late bacterial growth stage in vitro. Apparently, it was not easy to discern whether these genes were also expressed during colonization of the fungus. Five genes were identified as being specifically induced during colonization: Two corresponded to genes of unknown function, one of them encoded a protein similar to diacylglycerol kinase, which is involved in glucan synthesis in Rhizobiaceae (glucans seem to function during plant infection and adaptation to osmotic stress), the other two encoded an ABC transporter and an outer membrane porin, respectively.

4. CONCLUSIONS

Techniques based on whole genome sequence data (DNA arrays) seem to be displacing other more “traditional” ways of studying bacterial gene expression. However, the complexity of many environmental conditions limits the actual applicability of those techniques to the study of in vivo expression. The IVET system has proved a useful tool for a variety of complex experimental situations. Moreover, the answers that can be obtained by these two types of technique are of a different nature. Gene induction analysis with the IVET systems provides information at the level of single cell gene expression, since it is a particular clone carrying an IVET fusion active under certain conditions, the one that will be able to survive and be recovered. However, high throughput microarray analysis provides gene expression data relative to the total bacterial population. Comparative analysis of the results obtained with both methods will provide complementary knowledge. In vivo expression technology does not require a high number of cells, although an accurate representation of the genome in the library is crucial for the isolation of a wide number of *ivi* fusions.

One of the limitations of applying IVET to the study of gene expression is that it does not identify genes that are silent under the specific conditions of interest, although such genes may also be relevant to understand bacterial physiology in a particular environment. A bottleneck of the IVET approach, in our experience, is the confirmation of the *ivi* fusions by assaying the enzymatic activity encoded by the reporter gene in vivo. This problem is not always easy to solve, since the number of bacteria recovered from in vivo systems may be too low for a standard enzymatic assay. It is also important to remember that

induction in vivo does not necessarily mean that the gene plays a main role in infection. This is reflected by the fact that mutants in some loci were not affected in their plant infection ability (Table 3), although the existence of redundant functions may also account for the lack of effect.

Unexplored situations for the application of IVET in *Pseudomonas* are those involving abiotic stress conditions in nature, for example, saline stress, osmotic stress, heavy metals, etc. The identification of promoters that are preferentially induced under special conditions is also of major importance in bioremediation, by tradition a field of interest in suicide contained biosystems, where environmentally induced *ivi* promoters have obvious applications.

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LIFE STYLES

***PSEUDOMONAS* IN THE SOIL ENVIRONMENT**

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1. INTRODUCTION

The soil environment provides a maximum of ecological opportunity (vacant niche space) and competition, considered by Darwin²² to be the main causes of diversity patterns among species. Although this theoretical basis was not developed for microorganisms, it seems plausible that diversification among soil microorganisms including *Pseudomonas* spp. bacteria is indeed related to the heterogeneity of this environment. It has long been known that growth factors like availability of substrate, water and oxygen, etc. but also mortality factors like starvation and predation may vary within distances comparable to the microbial cell size. The bulk soil environment certainly carries a diversification of indigenous soil microorganisms on the surface and within aggregates, associated with pore structures or in water films. Yet, it is the active hot-spots of plant debris and the interactive plant–soil environments (spermosphere, rhizosphere and residuesphere) that have recently motivated

the microbiologists to work on soil *Pseudomonas* spp., perhaps bearing in mind the prospects of exploiting these bacteria for benefits of plant growth stimulation or protection, or for polluted-soil remediation.

In addition to the effect of ecological factors, intrinsic genetic factors of the specific soil microorganism are equally significant for their diversification⁹⁵. It is fair to say, that the molecular, single-cell or population level studies of soil *Pseudomonas* spp. during the past decade have contributed significantly to the advancement of modern soil microbiology in general. This progress has occurred despite the fact that bacteria show considerable ecological variation and still comprise a heterogenous group with constant need for revision of taxonomy and phylogeny. Major difficulties of defining the proper traits of distinction are presently found at the sub-generic level, perhaps understandable when considering that *Pseudomonas* spp. represent both aerobic and anaerobic energy metabolism, broad nutritional substrate spectrum, unique and abundant catabolic pathways, extensive secondary metabolite production, mobile gene pools and high environmental adaptability, elaborate sensory and regulatory circuits, and highly developed inter- and intrageneric communication.

Major limitations to the study of microbial function and diversity in soil was for long the lack of novel micro-scale and in situ technologies to differentiate specific populations and to observe their activity at the single-cell level. Over the past decade, however, a vast amount of new information on bacterial abundance, distribution and limiting factors of growth in the soil environment has been collected. Sørensen *et al.*¹⁰² summarized a number of approaches based on the modern technologies to study indigenous or inoculated *Pseudomonas* spp. populations in bulk soil and rhizosphere, for example, fluorescence stainings by cell surface-targeting antibodies or RNA-targeting oligonucleotide probes in combination with confocal laser scanning microscopy (CLSM), and gave specific examples on the use of inoculated *Pseudomonas* spp. strains (equipped with reporter genes) to provide information on specific, limiting factors (oxygen, nitrogen, phosphorous and iron availability) in bulk soil and rhizosphere environments.

This chapter will summarize the most important characteristics of soil *Pseudomonas* spp. populations and attempts will be made to identify specific selective factors supporting the large diversity of these bacteria in soil environments. Information will be based on both isolated *Pseudomonas* spp. (Sections 2 and 3), extracted soil DNA/RNA (Section 4) and on inoculated *Pseudomonas* spp. reporter strains (Section 5). Some cases representing possible selective systems will be dealt with, for example, the bulk soil including effects of water, soil compaction and organic pollution, but also the rhizosphere including effects of plant and soil type, oxygen and nutrient limitations.

2. HOW TO STUDY *PSEUDOMONAS* SPP. ISOLATES FROM SOIL

The wide distribution, functional diversity and significance, as well as the ease with which the *Pseudomonas* spp. can be cultured have undoubtedly been major reasons why the genus today constitutes one of the best-studied bacterial groups in soil. Over the last 10–20 years, attempts to isolate novel *Pseudomonas* strains with beneficial traits from soil have further been stimulated by their prosperous future as microbial inoculants in biotechnological applications. Culture-based techniques have also been popular to study root colonization and survival of specific inoculants and to compare population structures and their development among indigenous *Pseudomonas* spp. populations in the bulk soil and rhizosphere, respectively. Before some of the recent information on the diversity of culturable indigenous *Pseudomonas* spp. populations in soil is dealt with (Section 3), the following will present developments of the traditional isolation methodology used to culture pseudomonads, and of the assays used to further characterize isolates by phenotypic, chemotypic and genotypic assays.

2.1. Isolation Procedures

Pure cultures of new *Pseudomonas* species from soil can be obtained by variation of media and isolation procedures^{3, 46}. Nutrient-rich media such as King's B and Gould's S1 are traditionally used, but a recent study¹ demonstrated that unique *Pseudomonas* groups were obtained from soil using nutrient-poor media (cold soil extract) and that diversity among the isolates was dependent on Casamino acid levels in these media. Other authors^{39, 103} have shown that carbon and nutrient sources in soil-extract media play an important role for the diversity among soil isolates. Because soil is generally an oligotrophic environment, many *Pseudomonas* bacteria indigenous to the soil may even lack the ability to grow on the nutrient media tested so far. It seems clear that much can be gained in a continued effort to optimize isolation procedures for *Pseudomonas* from soil using nutrient-poor soil extracts modified by low supplements of carbon and nutrient sources, growth factors, osmolytes, etc.

2.2. Classification and Identification

In spite of modern molecular criteria, bacteria are today still classified according to their phenotypic traits, because such ranking often is readily made and has great practical significance⁸⁸. At best, such a classification provides information about the biological nature of the microorganism whereas

the molecular, phylogenetic information rather provides information about its evolutionary development. Any description of *Pseudomonas* spp. diversity in soil should thus be based on a polyphasic approach comprising both phenotypic and genotypic characters¹¹⁰. Some of the characteristic approaches used in the recent literature relating to *Pseudomonas* diversity in the soil environment will be presented briefly in the following.

2.2.1. Phenotypic Assays. Traditionally, the core of *Pseudomonas* taxonomy (species and biotype definitions) has been based on the ability of the isolates to utilize specific carbon compounds as the sole sources of carbon and energy^{76, 97}. A number of key characters such as UV-fluorescence (siderophore formation on iron-limited King's B or Gould's S1 media), arginine dihydrolase, denitrification and invertase (levan formation from sucrose) may thus give an early, tentative entry to the predominant group of saprophytic, fluorescent *Pseudomonas* spp. including *P. fluorescens* (biotypes I–VI) and *P. putida* (biotypes A–C)^{12, 100}. The subgroups all relate to the classical biotypes of Stanier *et al.*⁹⁷, except for the more recent *P. fluorescens* biotype VI and *P. putida* biotype C⁸; *P. fluorescens* biotypes I–V were thus Stanier biotypes A, B, C, F and G.

Such a limited number of nutritional characteristics may still provide a good basis for a tentative classification and identification of environmental *Pseudomonas* isolates to the species and biotype level. Unfortunately, it is often forgotten that such affiliations by phenotypic assays are indeed tentative—as was Stanier's proposal; his biotype F (here referred to as IV) was thus made on basis of only 2 strains! Not surprisingly and discussed briefly by examples in the following, numerous attempts have been made and different approaches have been tried to validate and improve the biotyping (subspecies) criteria, notably for *P. fluorescens* and *P. putida*. Even for commercial systems like Biotype 100 (bioMérieux, La Balme les Grottes, France) and Biolog MicroPlate (Biolog Inc, Hayward, United States), which generally provide similar clustering patterns, a number of *Pseudomonas* identifications have proven difficult^{12, 37}.

2.2.2. Chemotypic Assays. Chemometric fingerprinting methods are based on analysis of major cellular components as fatty acids, whole-cell proteins, lipopolysaccharides etc. The chemometric methods have been an attractive alternative to the phenotyping systems, because of speed and easy analysis protocols. A commercial system that provides the software support (MIDI) for species and, sometimes even subspecies identification of isolates by HPLC analysis of cellular fatty acid composition (fatty acid methyl ester, FAME derivatives) is now available. Several references provide evidence that selected peaks in whole-cell or phospholipid fatty acid profiles may identify *Pseudomonas sensu stricto*, but clearly contain insufficient information for a species or biotype separation^{42, 98, 108}.

Van Zyl and Steyn¹¹² found electrophoretic patterns of total soluble proteins useful to separate plant-pathogenic *Pseudomonas* spp. at the subspecies level. Intracellular protein patterns (AMBIS system) were similarly suggested to separate *P. fluorescens* biovars⁸⁴ while Lambert *et al.*⁵⁶ referred to protein electrotypes for whole-cell protein patterns of *Pseudomonas* spp. isolates from sugar beet rhizosphere. A more limited resolution has been obtained by analyses of selected bands in whole-cell, proteinase-K digested protein patterns, that appear to be useful for species separation¹⁰⁰, but not for resolution at the biovar level¹⁰⁹. Several years ago, Tesar and coworkers¹⁰⁵ introduced Westprinting as a method for identification of species of *Pseudomonas sensu stricto*. The method, which is not used extensively, relied on antisera that generated genus and species-specific immunological fingerprints by Western blot analysis. Westprinting could be used as a rapid screening assay as well as for identification of environmental isolates. The approach even allowed the identification of proteins conserved within *Pseudomonas* that could be recognized by monoclonal antibodies. In parallel Kragelund and coworkers prepared a polyclonal antibody against the conserved outer membrane porin OprF, which allowed specific identification of *Pseudomonas* isolates when used in a colony blot assay^{1, 53}.

The importance of electrophoretic LPS patterns to type *Pseudomonas* isolates has declined in recent years, but the method is still useful as demonstrated by Nielsen *et al.*⁷⁴ who used the approach to group isolates from sugar beet rhizosphere. Finally, while the potential of separating fluorescent *Pseudomonas* spp. by polyamine or quinone patterns¹⁵ was never exploited, there has been a recent and serious effort to classify the bacteria according to the fluorescent siderophore types; this approach holds great promise as the complicated chemical structures of these compounds are revealed¹⁴.

2.2.3. Genotypic Assays. Genetic fingerprinting assays have developed rapidly over the recent decade or so; from analysis of individual cultured isolates to detection in mixed, environmental samples; from analysis of whole populations to detection of single cells—from analysis of rDNA to detection by alternative genetic markers.

Current phylogenetic analysis of cultured isolates typically targets ribosomal DNA (rDNA), which is PCR-amplified by general (Bacteria) primers and submitted to subsequent restriction analysis (ARDRA) or sequencing. At present, directions of research are to introduce new groupings between genus and species (intrageneric groups, species complexes) and between species and clonal units (lineages, biotypes).

According to recent proposals^{73, 116}, *Pseudomonas sensu stricto* (rDNA group I, gamma-Proteobacteria, type species *P. aeruginosa*) has *c.* 30 valid species and may comprise two distinct intrageneric groups: (a) *P. aeruginosa* IGC1 with *c.* 10 species including the *P. aeruginosa* and *P. stutzeri* species complexes. (b) *P. fluorescens* IGC2 with *c.* 20 species including the *P. fluorescens*,

P. putida and *P. syringae* species complexes. As for the phenotypic biotyping described above the *P. fluorescens*–*P. putida* complexes still create the greatest problems even in the 16S rDNA-based phylogeny, using alternative phylogenetic markers (*gyrB*, DNA gyrase; *rpoD*, sigma70 factor), Yamamoto *et al.*¹¹⁶ suggested the *P. fluorescens* complex to be split into two lineages: (a) The *P. fluorescens* lineage containing most of the classical *P. fluorescens* biotypes (and other species). (b) The *P. chlororaphis* lineage containing *P. chlororaphis* (and former *P. aureofaciens*), *P. fluorescens* biotype V, *P. putida* biotype B but also the new *P. corrugata*. The latter group constitutes a hot-spot for difficulties of both phenotypic and phylogenetic classification and remains to be a source of misidentifications.

Specific *Pseudomonas* (sensu stricto)-selective PCR-primers for 16S rDNA amplification have been developed¹¹⁴ and subsequently used⁷⁹ in combination with restriction analysis (RFLP) to affiliate 14 forest and agricultural soil-derived clones and isolates into the 5 *Pseudomonas* species complexes (*P. aeruginosa*, *P. stutzeri*, *P. fluorescens*, *P. putida* and *P. syringae*) mentioned above.

Not only 16S rDNA, but also 23S rDNA and even the 16S–23S rDNA internal transcribed spacer (ITS) sequences have been in focus lately, again in combination with restriction analysis (e.g., ITS-RFLP). Christensen *et al.*¹⁹ found that 23S rDNA sequences of fluorescent *Pseudomonas* spp. were not useful to differentiate among *P. fluorescens* biotypes. While 16S rDNA (or 23S rDNA) based ARDRA, DGGE/TGGE or RFLP may only differentiate among *Pseudomonas* spp. at a relatively coarse (species) level of resolution, the ITS target appears to differentiate at a finer (“subspecies,” biotype) level where differentiation and taxonomy is most difficult (see above). Early ITS-targeting primer sets were designed by Kostman *et al.*⁵² and Jensen *et al.*⁴⁵. They were recently used in diversity studies of fluorescent *Pseudomonas* spp. in soil by Dawson *et al.*²³ and Cho and Tiedje¹⁷, respectively. A recent and interesting design by Locatelli *et al.*⁶² of *Pseudomonas*-specific primers targeting both the 16S rDNA (3' half) and the ITS region offers promising potential for characterization of soil *Pseudomonas* spp. populations, both at the species (16S rDNA) and subspecies (ITS) level.

For the highest possible resolution at the clonal (strain) level, cultivation may still be required—or detection should be made with highly specific antibody or oligonucleotide probes. To be mentioned here is that strain-level separation of cultivated soil *Pseudomonas* isolates are typically addressed by high-resolving fingerprinting of whole-genomic DNA. Among several PCR-based approaches, adopted for rapid identification and classification of soil bacterial isolates, the repetitive extragenic palindromic PCR (rep-PCR) using a selection of different primers (REP, BOX, ERIC) came to be the first of several important protocols developed²⁴. Even the RAPD-PCR (Random amplified polymorphic DNA-PCR) became widespread for typing purposes. Several applications of these PCR-fingerprinting protocols will be given in the following section.

2.3. Conclusions

Efforts to optimize isolation procedures for *Pseudomonas* from soil should be continued. Nutrient-poor soil extract media seem promising and could be supplemented with relevant carbon and nutrient sources, growth factors, as well as osmolytes etc. to protect against osmotic stress.

It is clear that much more testing of many more isolates is needed before a revised and perhaps final *Pseudomonas* spp. taxonomy is available. At present, the genotyping assays and thus the *Pseudomonas* spp. phylogeny advance quickly at both the intrageneric (species and species complexes) and the subspecies (lineage) level based on rDNA including ITS sequences or alternative genetic markers. Selective probes and primers for both in vitro and in situ identifications, targeting the *Pseudomonas* spp. at different levels of specificity, have been proposed, but should soon be supplemented as more environmental strains are being sequenced to reveal useful consensus patterns.

Undoubtedly, the phylogenetic work and development of genotyping assays will be setting both the speed and frame of reference for an ultimate (?) and long-awaited *Pseudomonas* spp. taxonomy. With such a prosperous goal, however, it will remain a challenge in parallel to develop better phenotypic assays (e.g., simple and robust, dichotomous keys based on a limited number of traits) or chemometric measures to distinguish the major groupings (species and subspecies/biotypes) for identifications of unknown soil isolates. Available commercial systems may already present important limitations and should thus be optimized and further developed; several cellular targets for such improvements seem not to have been fully exploited.

In soil microbiology, the functional diversity of microbial populations is obviously of utmost importance, but the complex *Pseudomonas* spp. taxonomy leading to tentative identifications (and often misidentifications) has long been an obstacle to the information obtained from isolation work. A polyphasic approach, using combinations of phenotypic, chemotypic and genotypic traits, will secure that the rapidly developing *Pseudomonas* spp. phylogeny and genotyping assays are matched by qualified, parallel information on the diversity based on functional traits.

3. DIVERSITY OF *PSEUDOMONAS* ISOLATES FROM SOIL

Fluorescent pseudomonads have a global distribution as judged from their frequent isolation from a number of most diverse soil environments. Recent developments of genotyping methodologies have allowed for new insight into local or regional distribution patterns, for example whether specific *Pseudomonas* groups are endemic or cosmopolitan. Cho and Tiedje¹⁷

recently demonstrated for fluorescent *Pseudomonas* spp. (*P. fluorescens*, *P. putida*, *P. aureofaciens*, *P. chlororaphis* and others) that 248 soil isolates showed strict endemism at the genotype level (rep-PCR genomic fingerprinting with BOX primers) but not at a coarser level of resolution (e.g., ARDRA). The endemism (high similarity between strains of a local site) in turn suggests a high degree of genomic diversity of fluorescent *Pseudomonas* and, hence, that geographical isolation plays an important role in bacterial diversification. An interesting observation was also that genetic distance among isolates based on the rep-PCR fingerprinting became smaller only when the geographic distance (between sampling sites) was relatively small. The study indicated that soil fluorescent *Pseudomonas* populations were endemic at the genotype level (at a scale of $<c.$ 200 km), but not at the coarser (subspecies) level.

Non-fluorescent *P. stutzeri* also has a cosmopolitan distribution and is found in marine and wastewaters, clinical samples and soil. A large strain collection from all these environments was recently studied by Sikorski *et al.*⁹⁰ using a variety of molecular fingerprinting procedures (e.g., RADP and Rep-PCR with BOX or ERIC primers); combined cluster analysis (UPGMA) separated the high genotypic diversity into seven groups which refined the 16S rDNA based phylogenetic grouping. In a subsequent study, Sikorski *et al.*⁹² demonstrated that a local soil population (120 strains) also had high genotypic diversity but could be clustered into distinct subpopulations using RADP with three different primers; interestingly, these subpopulations had characteristic levels of transformability with a broad-host range plasmid. This study indicates that local subpopulations may also be found for *P. stutzeri*, possibly evolving by differential rates of genetic change.

Several factors determine ecological and functional diversity among soil *Pseudomonas* species. However some eminent examples are those associated with exposure to xenobiotic compounds (catabolic traits) and with biotic interactions (rhizosphere and mycosphere signals). In the following, selected case studies will be presented to discuss the local environmental factors that may affect *Pseudomonas* spp. diversity in soil.

3.1. Case 1: *Pseudomonas* spp. in Degradation of Soil Pollutants

In polluted soil environments, *Pseudomonas* spp. populations are expectedly under strong selective pressure, favoring unique degradative capacity (growth metabolism) or toxicity resistance. Hence, a lower diversity resulting from the better survival of well-adapted populations may also be expected, often leading to isolation of both unique and novel strain groups. As discussed in the following examples taken from soils enriched with poly-aromatic

hydrocarbons (PAH) or the herbicide 2,4 dichlorophenoxyacetic acid (2,4-D), the complicated and yet unresolved plasmid ecology of indigenous *Pseudomonas* spp. populations probably harbour a key to how these populations develop in polluted soil environments.

3.1.1. Polycyclic Aromatic Hydrocarbons (PAH). A large number of *Pseudomonas* spp. capable of degrading PAH has been isolated from soil and aquifers^{50, 82}, but affiliations of the strains to taxonomic or phylogenetic groups have rarely been determined. Hence, it is not clear if the catabolic genes, which are chromosomal or plasmid-borne⁸⁶, are linked to other phenotypic traits of selective value in soil. Campbell *et al.*¹⁶ reported in a survey that a much elevated fraction (compared to agricultural soil) of fluorescent *Pseudomonas* spp. in both PAH-polluted (municipal coal gasification site) and metal-polluted (industrial steel works site) soils harbored plasmids, PAH (naphthalene and phenanthrene) degradation capability and resistance to heavy metals (Hg, Cu, Ni, Cd and Zn). Broad-host range, conjugative plasmids would be likely to carry the PAH-degradative genes in indigenous *Pseudomonas* spp. populations in polluted soils; however, Campbell *et al.*¹⁶ were unable to find the IncP-type most commonly associated with PAH degradation¹¹. Although PAH degradation could not be immediately verified by presence of a particular plasmid size or compatibility type among the indigenous *Pseudomonas* spp. isolates from soil, the role of conjugative plasmids in local and rapid selection of unique populations could well be masked by the chromosomal integration of degradation genes.

Unique traits like degradation or toxicity resistance in the polluted soil environment should promote a progress toward purifying selection and thus a low population diversity. Johnsen *et al.*⁴⁶ isolated 41 phenanthrene-degrading *Pseudomonas* sp. from the gasification site studied by Campbell *et al.*¹⁶ and a large majority of isolates could be tentatively assigned to *P. fluorescens* biotypes II, III and IV by classical phenotypic tests. A subgroup of strains initially affiliated to *P. fluorescens* biotype III was later reassigned to a new species, *P. frederiksbergensis*³ with similarity to the *P. fluorescens* species complex (including *P. chlororaphis* and *P. corrugata*) but also to the more distantly related *P. syringae* species (including *P. amygdali*). This work indicates that selection may locally propagate a unique *Pseudomonas* spp. population in PAH-polluted soil environment, distinguishable even at the species level.

3.1.2. Herbicides. Ironically, degradation of the most commonly studied herbicides, phenoxyacetic acids such as 2,4-D, is now primarily associated with former *Pseudomonas* species, for example, *Burkholderia (cepacia)*, *Comamonas (acidovorans)*, *Ralstonia (solanacearum)* and *Sphingomonas (paucimobilis)*. Ka *et al.*⁴⁸ characterized the diversity of degrading isolates

from 2,4-D treated field soil and found that *P. pickettii* and *P. solanacearum* were involved; however, both have later been reclassified as non-*Pseudomonas* spp. Still, genuine *Pseudomonas* spp. could be involved if the *tfd* genes of 2,4-D degradation were transferred on broad-host-range conjugative (e.g., IncP1-type) plasmids readily received by *P. fluorescens* and *P. putida*²⁵; The competitiveness of *P. chlororaphis* carrying such a 2,4-D degradative plasmid (pJP4) has recently been studied in rhizosphere soil⁸⁹. McGowen *et al.*⁷⁰ provided evidence for the role of interspecies gene transfer in the evolution of 2,4-D degraders, but also pointed out that some broad-host range plasmids like pJP4 may not express the 2,4-D pathway when transferred to common *Pseudomonas* species (e.g., *P. fluorescens*, *P. putida*). Phylogenetically limited expression of certain, plasmid-borne chlorobenzoate-degradative genes has also been noted for the *Pseudomonas* spp.⁵³.

3.2. Case 2: *Pseudomonas* spp. in Rhizosphere Development

Recruitment of saprophytic rhizosphere microbial communities from the indigenous bulk soil microflora has long been of interest, since this process may often be quite selective leading to a higher density or a particular composition of microorganisms at the root–soil interface. Much effort has lately been applied to find the key determinants of selective root colonization by seed-inoculated *Pseudomonas* spp. strains⁶³ while less attention have been devoted to colonization by the indigenous soil populations. Comparison of rhizosphere and bulk soil *Pseudomonas* spp. diversities is one such approach to identify possible, selective traits for root colonization. However, as shown in following case studies in different plant rhizosphere–bulk soil systems, there is a strong influence of soil type and plant species/cultivar.

3.2.1. Selection of “*P. fluorescens*”. Before the PCR-based fingerprinting techniques became available approx. 10 years ago, several studies had already indicated a diversification of the isolates of saprophytic, fluorescent *Pseudomonas* spp. from rhizosphere and bulk soil samples, respectively, as judged from a few phenotypic traits. The classical reference is the work of Sands and Rovira⁸⁵ demonstrating a predominance of *P. fluorescens* biotype G (subsequently referred to as biotype V) in Australian soil and wheat rhizosphere. This biotype (together with *P. putida* biotype B) was long considered as “intermediate” and “difficult” groups within the *P. fluorescens*–*P. putida* species complex, waiting for reclassification and redefinition. Much later, when ARDRA profiling had become available, Ross *et al.*⁸³ affiliated a majority of *Pseudomonas* isolates from Australian wheat field soil into *P. corrugata*. Achouak *et al.*² studying *P. corrugata* in French agricultural soil confirmed

that ARDRA patterns could affiliate soil isolates to this species, which may then be difficult to discern only by the classical phenotypic tests. Achouak and coworkers could even discriminate an impact of crop management (crop rotation versus wheat monoculture) at the genotype level (genotypic diversity) for *P. corrugata* as analysed by rep-PCR fingerprinting (with ERIC primers).

Early indications of selection among indigenous *Pseudomonas* spp. populations during root colonization came from groupings performed according to electrophoretic whole-cell protein profiles, referred to as protein electrotypes (Lambert *et al.*)^{56, 57}. These two studies described abundances and diversities of rhizobacterial *Pseudomonas* spp. populations on maize and sugar beet roots and conclusions were made about selective enrichments of the electrotypes (resolving taxonomically at the strain level) on single plant individuals, among different cultivars and within different fields. For instance, an abundance of early-colonizing *P. fluorescens* in sugar beet rhizosphere was noticed, including a high, intraspecies diversity when the isolates came from different field plots or stages of seedling development. Further assignment of the electrotypes to specific *P. fluorescens* biotypes was not made in these studies.

Some years later, a first and suitable example of directed search for soil factors determining preferential selection among biotypes within the *P. fluorescens* species is the work by Frey *et al.*³². These authors found that a majority (90%) of 300 isolates from bulk soil and Douglas fir seedling roots (infested with mycorrhiza fungus, *Lactaria bicolor*) could be assigned to this species. Interestingly, almost all isolates from the bulk soil gathered in *P. fluorescens* were biotypes II and V, while isolates from the rhizosphere were distributed equally in biotype I and biotypes II and V, respectively. It was suggested that the mycorrhiza-infested rhizosphere exerted a selective stimulation of *P. fluorescens* biotype I at the expense of biotype V, controlled by trehalose production in the mycorrhizae.

The work of de Bruijn²⁴, actually emphasizing and elaborating mostly on *Rhizobium* spp., presented the first rep-PCR fingerprint patterns of soil *Pseudomonas* spp. isolates. In the next years to come, rep-PCR was the method of choice for genomic diversity studies of soil fluorescent *Pseudomonas* spp. isolates, and comparisons of phenetic and genotypic fingerprinting patterns were carried out. Suitable examples are the studies in French soils by Lemanceau and coworkers. In the first work in heavy clay-silt Dijon soil⁶⁰ phenotypic characterizations (primarily substrate utilization patterns) were shown to be well correlated with genotypic patterns based on rep-PCR; several biotypes of the *P. fluorescens*-*P. putida* species complex were found in the soil and rhizosphere samples, but *P. fluorescens* biotype II and *P. putida* biotype A were preferentially abundant in tomato and flax rhizospheres, respectively. The subsequent study⁵⁹ in calcic (c. 40% CaCO₃) Chateaufort soil generally confirmed the above observations, but further stressed the

significance of soil type on the outcome of selected rhizosphere populations; here, *P. putida* biotype A was predominant in the uncultivated soil, but *P. fluorescens* biotype II became most abundant in the tomato and flax rhizospheres. Biotype II is one of the denitrifying biotypes (II, III and IV) and this phenotype may actually be selected for in the tomato and flax rhizosphere as shown in the studies by Clays-Josserand *et al.*^{20,21}. In the former work, *P. fluorescens* and *P. putida* were again the most abundant species in root compartment and bulk soil samples, respectively; the denitrifying strains (biotypes II–IV) comprised a higher proportion in root tissue (94%), rhizoplane (75%) and rhizosphere (68%) than in the uncultivated bulk soil (44%). Even earlier, Linne von Berg and Bothe⁶¹ had suggested that denitrification has a selective advantage at roots. From the studies, it appears that the denitrification phenotype (viz. anaerobic respiration) may contribute to differences in both species and subspecies (biotype) composition of fluorescent *Pseudomonas* spp. between root and soil compartments.

3.2.2. Selection of other *Pseudomonas* spp. The last rhizosphere system to be dealt with here is that of Cruciferan plants like oilseed rape, which appear to be a source of particular *Pseudomonas* spp. groups. For instance, in the German agricultural soil studied by Berg *et al.*⁹, strong selection was seen for *P. chlororaphis* in oilseed rape rhizosphere; as in the French soils referred to above, *Pseudomonas putida* biotype A was also predominant in German agricultural soil. Unfortunately, the fatty acid methyl ester (FAME) analysis and MIDI strain identification did not allow Berg *et al.*⁹ to distinguish between *P. fluorescens* biotypes. Supporting evidence for *P. chlororaphis* being a predominant species in oilseed rape rhizosphere came from several concurrent studies in US soil by Germida and coworkers⁷².

Another example of Cruciferan rhizosphere being a source of new *Pseudomonas* spp. species is the new *P. brassicacearum* isolated in French soil². Interestingly, a local population of this new species in German soil was recently shown at high resolution (RAPD-PCR) to consist of several subgroups (referred to as lineages), one of which dominated strongly⁹¹. Detailed data analysis indicated that the local population had developed under strong purifying selection.

3.3. Case 3: *Pseudomonas* spp. in Fungal Antagonism

Many fluorescent *Pseudomonas* spp. strains are capable of reducing the incidence of plant diseases caused by soil-borne fungi and a number of studies have examined the taxonomic and functional diversity of these bacteria. Production of biosurfactants for example, cyclic lipopeptides (CLP) and antifungal metabolites such as diacetyl-phloroglucinol (DAPG), pyoluteorin (PLT) and cyanide (HCN) has been studied most intensively. The ecological

functions of secondary metabolites are often still unclear, however. In the present context, the recent attempts to relate secondary metabolite production to population diversity in plant spermosphere/rhizosphere and disease-suppressive soils are indeed interesting. A major contribution and source of inspiration is here the studies of antibiotic (DAPG)-producing *Pseudomonas* spp. and their apparently high frequency of occurrence in disease-suppressive soils (Section 3.3.2.).

3.3.1. Selection of CLP-Producing *Pseudomonas* spp. Sugar beet rhizosphere has been much studied in relation to biocontrol of important pathogenic microfungi, and indigenous soil *Pseudomonas* spp. populations are important sources of fungal antagonists. In a recent survey in Danish agricultural fields, Nielsen *et al.*⁷⁵ searched for *Pseudomonas* spp. isolates producing cyclic lipopeptides (CLP) acting as both biosurfactants and antifungal agents. The authors found a predominance of *P. fluorescens* biotype I and VI among the early root colonizers on sugar beet seedlings grown in Højbakkegaard sandy loam. Hence, in this rhizosphere system, the denitrifying *P. fluorescens* biotypes (II, III and IV) were uncommon. As shown below, this may be different in other agricultural systems, probably determined by soil type and cropping. Interestingly, the relatively new *P. fluorescens* biotype VI (close to biotype I) introduced by Barrett *et al.*⁸ and recorded at an early stage in cucumber rhizosphere by Sørensen *et al.*¹⁰⁰ was also relatively abundant in French soil, including tomato and flax rhizospheres⁵⁹. As was the case for *P. corrugata* isolated in both Australian⁸³ and French² soil, *P. fluorescens* biotype VI may be widely distributed and probably cosmopolitan. As mentioned earlier for *P. corrugata*, better detection techniques such as dichotomous keys¹² must be developed for new groups such as *P. fluorescens* biotype VI; one problem is that the phenotypic characteristics of these groups are often a loss of one or several of the “typical” *Pseudomonas* traits (e.g., catabolism of single sugar monomers, alcohols and amino acids), rather than presence or absence of robust biotype traits as denitrification characterizing *P. fluorescens* biotypes II, III and IV.

3.3.2. Selection of DAPG-Producing *Pseudomonas* spp. An important field of study has been the diversity of DAPG-producing fluorescent *Pseudomonas* spp. in disease-suppressive soils initiated by Weller and coworkers. Comparing strain collections (total c. 225 strains) originating from soils from different geographic locations worldwide^{35, 49, 58, 77, 113}, the following patterns were evident: The strains could be separated into only 2–3 predominant ARDRA groups of which the largest one always contained the reference biocontrol strain Q2–87, belonging to *P. fluorescens* biotype II according to Raaijmakers and Weller⁸⁰. A smaller ARDRA group contained the reference biocontrol strains CHA0 and Pf-5 also producing PLT and belonging to

P. putida A according to FAME analysis²⁸: A third and very small ARDRA group contained the biocontrol reference strain F113. The largest ARDRA group largely represented one phenotype (Biolog) and was presumably a homogenous collection of *P. fluorescens* including biotype II. In Danish soil, Nielsen *et al.*⁷⁴ also found DAPG production to be associated with *P. fluorescens* biotype II. In the study by Raaijmakers and Weller⁸⁰, further genotyping of the isolates was made by RAPD, rep-PCR (both BOX and ERIC primers) and RFLP, the assays indicating a total of 10–20 genotypes different at the strain level. In the study of Picard *et al.*⁷⁷, strain diversity (RAPD-PCR fingerprinting) was lowest on the root surface or rhizoplane (washed root sample) and during early root development, highest in the rhizosphere soil and generally increasing with root age. Development of the early-colonizing population of DAPG-producing *P. fluorescens* was proposed to link to root exudate production, but DAPG production was unlikely to be selective per se.

3.4. Conclusions

In spite of a high degree of genomic diversity of fluorescent *Pseudomonas*, available data indicates that local subpopulations occur and that geographical isolation plays an important role in *Pseudomonas* diversification. In addition a few factors that may be important for the local selection of unique *Pseudomonas* populations have been identified. For bulk soil environments pollution with PAH compounds is one such example.

For rhizosphere environments more general information is available, although this information appear as fragmented case studies addressing different plant species established in different soil types. As shown in Table 1, the ability of tomato, flax and oilseed rape rhizosphere to select specific *P. fluorescens*, *P. putida* and *P. chlororaphis* biotypes is well documented, although the significance of the soil type should not be neglected. Furthermore mycorrhiza-infested rhizosphere seems to select for a specific *P. fluorescens* biotype at the expense of another.

It remains to be seen if the plant rhizosphere in general is indeed strongly selective to particular *Pseudomonas* spp. groups, and if so, what are the controlling factors? Do plant groups known to exude unique compounds also harbor unique *Pseudomonas* spp. populations in their rhizosphere? In combination with phenotypic traits characterizing the isolates, the high-resolution chemotyping and genotyping methods should continue to assist us in identifying the specific levels of selection among indigenous *Pseudomonas* spp. populations in soil, be it at the species or at a finer subspecies level. Efforts should continue to verify if the functional traits operating to select among indigenous populations in soil are also those being used presently to distinguish and group them taxonomically.

Table 1. Predominant fluorescent *Pseudomonas* spp. and biotypes isolated from agricultural soil and rhizosphere samples (publications 1995–2002).

	Species and biotype	References
Bulk soil	<i>P. putida</i> A	[9, 20, 59]
Sugar beet	<i>P. fluorescens</i> I, VI	[75]
Tomato	<i>P. fluorescens</i> II	[59, 60]
Flax	<i>P. fluorescens</i> II	[60]
	<i>P. putida</i> A	[59]
Wheat	<i>P. corrugata</i>	[2, 83]
Oilseed rape	<i>P. brassicacearum</i>	[2]
	<i>P. chlororaphis</i>	[9, 72]

Hence, while general conclusions may be difficult to draw, the frequent predominance of specific *Pseudomonas* species or biotypes within the many different experimental systems probably indicate that selection may indeed occur on basis of the phenotypic traits commonly considered to separate the *Pseudomonas* spp. at the species/subspecies level.

The ability to produce secondary metabolites—variable within the species or biotype—is in some cases predominant in soil *Pseudomonas* populations. Whether the antagonistic trait, for example, production of an antifungal metabolite is selective per se may be doubtful. One reason could be that secondary metabolite production is often co-regulated with other physiological traits that mask a direct selectivity. However, the frequent recovery of CLP- or DAPG-producing *Pseudomonas* strains in specific plant rhizospheres or in specific fungal disease-suppressive soils is a strong stimulus for further search and discovery of the truly selective factors in these communities.

4. DIVERSITY OF *PSEUDOMONAS* DNA/RNA IN SOIL

There is strong evidence that identification of pure cultures on laboratory media detects only 1% or so of the bacteria present, due to the selectivity of growth media and incubation conditions. This continues to be a great concern and there is an urgent need to compare the classical isolations and the new molecular methods to see if this discrepancy is valid for all bacterial groups. As described below there is still hope that *Pseudomonas* may comprise a group where environmental populations are easily culturable and thus provides an exception to the difficulties encountered for other groups. Still, it is clearly

important to develop specific molecular assays for *Pseudomonas* community analysis directly in soil samples, as described in the following. While the assays described below are based on ribosomal DNA/RNA analysis, it should be mentioned that alternative targets among functional genes, for example, the alkaline metallopeptidase (*apr*) genes specific for the *P. fluorescens* biotypes⁴ may be useful for community analysis in soil⁵.

4.1. In situ rDNA/RNA Analysis of *Pseudomonas* Community

As improvements of soil DNA extraction and subsequent PCR, fragment separation, cloning and sequencing will follow the general development for other phylogenetic groups, the major specific limitation to *Pseudomonas* studies continues to be the phylogenetic complexity of this group. Hence, difficulties of nucleotide probe and primer design adequately explain why such studies are still relatively few, compared to several recent reports on phylogenetically well-defined soil bacteria such as the beta-Proteobacterial ammonia oxidizers.

While the partial or full 16S rDNA sequence is a basis for phylogenetic identification of *Pseudomonas* sp. *sensu strictu*, there is yet limited evidence that short oligonucleotide probes or PCR primers may be adequately specific to cover this phylogenetic group. The 16S-probe PSM_G¹³ has been used by Marilley and Aragno⁶⁵ to verify affiliation of unknown soil DNA to *Pseudomonas* spp. (Section 4.3.2.). Similarly, the 23S-probes Ps and Ppu may be used in combination to distinguish members of the genuine *Pseudomonas* spp. from other bacteria⁸⁷.

By comparison, there have been more attempts to define *Pseudomonas* spp. primers for PCR amplification covering the whole or smaller subgroups with “general” or “specific” primers, respectively. One strategy is here to define one of the primers in a strongly conserved region, the other in a variable, but *Pseudomonas*-specific region of the 16S rDNA. Johnsen *et al.*⁴⁷ used the PSM_G probe described above as the *Pseudomonas*-specific primer (together with a general eubacterial primer) in a quantitative PCR to study population dynamics of indigenous *Pseudomonas* spp. in soil. Alignments of the 16S rDNA representing a super-species (lineage) grouping of the fluorescent *Pseudomonas* spp. suggest that regions may exist for suitable primer design, but so far this has not been implemented. Similarly, efforts to find alternative PCR primers designed within the large-subunit 23S rDNA (Section 2.2.3.) have so far not been successful enough in the complex environmental communities. Alternative to the search for specific *Pseudomonas* spp. primers, a most promising strategy has been to PCR-amplify environmental (soil) DNA

by general eubacterial 16S rDNA primers for a whole-community fingerprinting followed by a specific verification of the *Pseudomonas* spp. population. Here, a clone library for sequencing is established directly from the original PCR product or from excised DNA fragments (gel bands) separated by for example, community ARDRA⁶ or DGGE²⁹ techniques.

At the present, early stage of molecular methods development, polluted soils or rhizosphere have been suitable objects for studies of *Pseudomonas* spp. populations. One reason is that short-term community changes or even strong selection and enrichment for *Pseudomonas* spp. subpopulations are likely after soil treatment with PAH or herbicides, and in the rhizosphere. Another one is that the early trials of the new methods based on soil DNA/RNA analysis (e.g., community ARDRA and DGGE/TGGE) and subsequent identification of dominating bands have been limited to dominating populations and have only been semi-quantitative. The following Sections 4.2 and 4.3. present selected case studies to exemplify this new research on *Pseudomonas* spp. community structure in soil and rhizosphere environments.

4.2. Case 1: Bulk Soil Including Polluted Soil Environments

4.2.1. PAH. The recent work of Madsen and coworkers may serve to illustrate the great progress being made, but also the current limitations, using molecular (DNA/RNA based) methods to reveal both community structure and in situ activity in soil environments. Groundwater from a coal tar waste-contaminated site was investigated using ARDRA community analysis⁶, but the 16S rDNA patterns did not reveal a predominance of *Pseudomonas* spp. However, when naphthalene dioxygenase gene (*nahAc*) transcripts (mRNA) extracted from the groundwater was reverse-transcribed and amplified, two major products (as revealed by subsequent cloning and sequencing) were related to *Pseudomonas* spp. and *Burkholderia* strains, respectively¹¹⁵. Another advanced and recent study by the group⁷ was the use of a fluorescent oligonucleotide probe (designed from sequences harboured in a *P. putida* strain) to target (FISH) the bacteria with naphthalene dioxygenase mRNA transcripts in natural samples; tyramide signal amplification, chilled-CCD camera detection and extended exposure gave sufficient CY3-fluorescence for single-cell observations in the microscope. A realistic, c. 1% of the total bacterial population contained naphthalene dioxygenase gene transcripts, although probe specificity did not resolve *Pseudomonas* spp., *Burkholderia* spp. and *Comamonas* (former *Pseudomonas*) *testosteroni nahAc* genes.

4.2.2. Herbicides. Both present and former *Pseudomonas* species are likely to play important roles and be selective enriched during herbicide degradations in soil. El Fantroussi *et al.*²⁹ made DGGE analysis of community 16S rDNA in soil after long-term treatment with Linuron (phenyl urea derivative) herbicide; 4 of c. 10 bands appearing after fresh enrichment with Linuron in such soil samples were cloned and assigned to *Pseudomonas* spp. (*P. fluorescens*, *P. putida*, *P. mandelii* and *P. jessenii*) after sequencing. The predominance of *Pseudomonas* spp. enriched by Linuron is in contrast to the observations by Engelen *et al.*³⁰ who used TGGE of community 16S rDNA in search for populations specifically enriched after treatment with Herbogil (Dinoterb, 2-tert-Butyl-4,6-dinitrophenol) herbicide; one of the two distinct bands appearing in herbicide-treated soil were cloned and assigned to *Xanthomonas* spp. after sequencing. Finally, 2,4-D was selected in Section 3.1.2. to give an example of a common phenoxyacetic acid-type herbicide, for which the role of genuine *Pseudomonas* spp. in degradation is still uncertain. Tiedje *et al.*¹⁰⁶ recently gave an example of ARDRA-based community analysis of DNA extracted from 2,4-D treated soil; the only two distinct bands appearing were assigned to *Burkholderia* spp.

4.3. Case 2: The Rhizosphere Environment

4.3.1. Long-Term Rhizosphere Communities. McCaig *et al.*⁶⁸ performed 16S rDNA sequence analysis of total DNA extracted from Scottish permanent grassland soil, the PCR products (bacterial primers) were subsequently cloned and partially sequenced for phylogenetic analysis. Of all 275 clones, only 11 (4%) were gamma-Proteobacteria, and only 2 of these clones (<1% of total) were genuine fluorescent *Pseudomonas* spp. as judged from their close sequence homology to well-known culturable strains. In a subsequent study McCaig *et al.*⁶⁹ made a partial 16S rDNA sequencing on 12 isolates from *Pseudomonas* isolation agar and found that 11 were gamma-Proteobacteria and 10 of these were close related to the two environmental *Pseudomonas* clones and to well-known culturable *Pseudomonas* species (e.g., saprophytic *P. fluorescens* and *P. corrugata* or the pathogenic *P. veronii* and *P. tolaasii*). Despite their relative infrequency in this permanent grassland soil, the environmental *Pseudomonas* clones all had a high sequence homology to well-known, culturable *Pseudomonas* species.

Low abundance of gamma-Proteobacteria and *Pseudomonas* was also observed in Canadian pine forest soil (*Pinus contorta* rhizosphere) as studied by Chow *et al.*¹⁸. Among a total of 709 clones, 64 (9%) were gamma-Proteobacteria, and only 6 of the clones (<1% of total) could be ascribed to fluorescent *Pseudomonas* spp. according to Genbank homologies.

Another comparison of molecular and culture-based analyses was made by Furlong *et al.*³⁴ in US no-till agricultural soil, c. 100 clones from both bulk soil and earthworm (*Lumbricus rubellus*) casts, respectively, and 230 isolates were compared. While the abundances of many of the taxa in soil and cast clone libraries were similar, the abundance of other taxa changed dramatically. For instance, gamma-Proteobacteria clones increased from 4% of the soil library to 22% of the cast library, indicating that nutritional replenishment during gut passage was stimulatory to this phylogenetic group. The large majority of the gamma-Proteobacteria clones were in turn closely related to well-known culturable *Pseudomonas* species (e.g., *P. fluorescens* and *P. putida* or *P. veronii*). Most of the soil gamma-Proteobacteria isolates were also associated with the family Pseudomonadaceae and together with the molecular evidence, this study suggests that culturable *Pseudomonas* may represent a significant fraction of the total *Pseudomonas* population in the soil.

4.3.2. Short-Term Rhizosphere Community. Several examples of in situ selection-enrichment for *Pseudomonas* spp. in rhizosphere soil of agricultural crops were given above, based on identification of isolates (Section 3). However, the molecular detection of *Pseudomonas* spp. among environmental 16S rDNA clones is confirmative to these observations. Smit *et al.*⁹⁴ studied the abundance of *Pseudomonas* in wheat rhizosphere of Dutch agricultural soil and found the gamma-Proteobacteria to comprise 10% and 16% of soil isolates (1/10 TSA) and rDNA clones, respectively. Fluorescent *Pseudomonas* spp. were the predominant gamma-Proteobacteria as judged from FAME identification of the isolates.

A final example showing the stimulatory effect on soil *Pseudomonas* populations by rhizosphere of agricultural crops is the work by Marilley *et al.*⁶⁶. Here, the number of clones phylogenetically related to *Pseudomonas* spp. were higher in both *Lolium perenne* (ryegrass) and *Trifolium repens* (white clover) rhizosphere soil compared to bulk soil, clone sequencing was not performed in this study, but affiliation to *Pseudomonas* sp. was demonstrated by colony hybridization using the *Pseudomonas*-specific PSM_G oligonucleotide probe. In a parallel study⁶⁵ based on partial sequencing of the 16S rDNA clones, the plant roots were shown to have a selective effect towards the gamma-Proteobacteria leading to a predominance of *Pseudomonas* spp.

Duineveld *et al.*²⁶ made an interesting attempt to compare the relative abundances of 16S rDNA and corresponding 16S rRNA (after RT-PCR) fragments in *Chrysanthemum* rhizosphere soil. Prominent PCR products were separated by DGGE and excised for sequencing in order to identify both active and total populations as revealed by abundant 16S rRNA and 16S rDNA fragments, respectively. The rDNA analysis demonstrated fewer DGGE bands (lower diversity) in the rhizosphere and *Pseudomonas* spp. were among the

abundant genera as judged from the database homologies obtained. Moreover, apart from several *Bacillus*-related bands, at last three (out of 12) of the 16S rRNA bands were related to *Pseudomonas* spp.

4.4. Conclusions

As has been the case also for studies based on soil isolates, the phylogenetic complexity of *Pseudomonas* spp. has so far been a major limitation to the molecular, DNA/RNA-based studies of their abundance and diversity in soil samples. As a result, there are yet relatively few oligonucleotide probes and PCR primers available for direct and specific detection of *Pseudomonas* spp. in such environmental samples. The common solution has so far been a general PCR amplification of soil DNA by Eubacterial primers, followed by specific verification of the *Pseudomonas* spp. population using a cloning-and-sequencing protocol.

The first molecular attempts to find effects by soil pollutants on natural bacterial diversity have reported that *Pseudomonas* spp. rDNA may occasionally appear among the prominent PCR amplicons (ARDRA or DGGE bands on separation gels), taken to represent a differential enrichment of these bacteria. Such population effects may expectedly depend on the pollutant compound, in addition to the indigenous soil type, clearly, the role of *Pseudomonas* spp. in pollutant degradation in soil is yet unclear and should gain from more molecular studies at field sites with a well-described pollutant history.

In a variety of soil systems, the DNA/RNA-based methods have already provided several excellent studies of *Pseudomonas* spp. abundance and diversification. As shown in Table 2, bulk soil and long-term rhizosphere

Table 2. Frequences of gamma-Proteobacteria and *Pseudomonas* spp. in soil and rhizosphere samples based on sequence analysis of environmental 16S rDNA clones.

	Gamma-Proteobacteria (%)	<i>Pseudomonas</i> spp. (%)	References
Forest soil	9	<1	[18]
Grassland soil	4	<1	[68]
No-till soil	4	<4 ^a	[34]
Earthworm cast soil	22	<22 ^a	[34]
Wheat rhizosphere	16	<16 ^a	[94]
Ryegrass/clover rhizosphere	ND	20	[66]
<i>Chrysanthemum</i> rhizosphere	ND	25 ^b	[26]

ND Not determined.

^aData indicates majority of gamma-Proteobacteria.

^bData based on 16S rRNA.

environments (e.g., permanent pasture, forest and no-till agricultural soils) the *Pseudomonas* spp. population may comprise a relatively small fraction (1–4%) of rDNA clones obtained after initial PCR amplification using Eubacterial primers; in contrast, earthworm casts and short-term rhizosphere communities (agricultural crops) may harbor a larger fraction (16%–25%). Taken that this information is still based on relatively few studies and represent a diverse array of soil types and individual protocols, the results are still sensible and in accordance with expected diversification in low-C and high-C input soil systems, respectively.

5. LIFE CONDITIONS FOR *PSEUDOMONAS* SPP. IN SOIL AND RHIZOSPHERE

Marker genes are “genes that confer the tagged strain with an easily detectable, continuously expressed phenotype enabling specific monitoring of the strain.”⁴³ Tagging of *Pseudomonas* spp. strains with marker genes has provided a means of single-cell visualization in the soil environment, for example, micro-localization on plant roots and spatial relations with other microbial populations. A parallel achievement has been the construction of *Pseudomonas* spp. strains carrying environmentally regulated reporter genes for studies of specific gene expression in situ. Reporter gene technologies typically rely on analyses of one selected, genetically modified strain at a time, but this reporter strain may provide indicative information on for example, the limiting factors for a *Pseudomonas* spp. community in the soil or rhizosphere samples. Sørensen¹⁰¹ and Sørensen *et al.*¹⁰² recently reviewed the novel information obtained by marker and reporter gene techniques on the distribution and activity of pseudomonads in soil and rhizosphere habitats. A few applications of these technologies to determine single-cell localization, growth, metabolic activity and limiting or stimulating factors are briefly highlighted in the following, together with some recent applications to study the advanced, molecular signalling and communication within the *Pseudomonas* spp. community in soil.

5.1. Distribution, Growth and Metabolic Activity

Monitoring of bioluminescence from *lux*-tagged strains has been an early, much-adopted marker gene technology to detect populations of *Pseudomonas* spp. in soil microhabitats. Depending on the construct and using a sensitive CCD camera, bioluminescence is typically high enough to detect

the overall distribution patterns of cells, but may often be inadequate to visualize each single cell⁵⁴. Subsequently, detailed images of seed-inoculated *Pseudomonas* spp. strains in rhizosphere has been obtained by immunofluorescence combined with CLSM³⁸ and fluorescence microscopy of *gfp*-tagged cells^{10, 81}. Important observations have been the preferential localization of proliferating inoculant cells (microcolonies) in the crevices between root epithelial cells or on root hairs. The studies have further indicated that active migration of inoculant cells between the root surface and the surrounding mucigel or sloughed-off cells may occur^{38, 81}. Finally, interactions between inoculant and indigenous bacteria during root colonization have been studied using CLSM; one example is here the study of Lübeck *et al.*⁶⁴ showing that seed-inoculated *Pseudomonas* spp. cells on the young sugar beet roots may delay colonization by indigenous soil bacteria.

The application of reporter gene technology to study *Pseudomonas* spp. growth activity in their environment may be illustrated by the work of Marschner and Crowley⁶⁷, who used a ribosomal promoter-driven *lux* reporter (emitting bioluminescence during growth, when rDNA genes are highly expressed); the authors reported that growth activity of a *P. fluorescens* strain was higher in natural rhizosphere (pepper) than in bulk soil. Ramos *et al.*⁸¹, using a ribosomal promoter-driven *gfp* reporter (unstable Gfp variant) in a *P. putida* strain, found that growth activity was only detectable gnotobiotic rhizosphere (barley) and only near the root tip. The presence of a large majority of non-growing *Pseudomonas* spp. cells on the root surfaces after seed inoculation was confirmed by Lübeck *et al.*⁶⁴ who detected rRNA contents by direct fluorescence in situ hybridization (FISH) staining of the sugar beet root specimen, growth-active bacteria were detected as microcolonies of dividing cells, occurring between the plant epithelial cells at the root basis or at the emerging, secondary roots or root hairs.

Alternative to cell division (growth activity), essential metabolic activity within single bacteria may also be used to monitor *Pseudomonas* spp. populations in soil and rhizosphere environments. The classical example is the application of a *lux* gene cassette under control of a constitutively expressed promoter. The bioluminescence signal is here solely dependent on cellular metabolic activity (NADH⁺ generation) when oxygen and the substrate aldehyde (decanal) for luciferase enzyme activity are provided in surplus. Meikle *et al.*⁷¹ using such a *lux* system in *P. fluorescens* strains reported loss of metabolic activity in drying soils. Kragelund *et al.*⁵⁴ found that metabolic activity in a root-colonizing *P. fluorescens* strain varied along the root and was different on the root surface (rhizoplane) compared to the surrounding rhizosphere. Finally, Unge *et al.*¹⁰⁷ presented a dual *gfp-lux* system in soil-inoculated *P. fluorescens*, providing detailed images of both cellular localization (*gfp*) and metabolic activity (*lux*).

5.2. Nutrient Availability

Roots exudates have long been considered to be the major C source supporting growth of root-colonizing bacteria in the young rhizosphere. A recent development has been the use of *lux*-reporter (bioluminescence) gene-equipped model bacteria to detect the actual C-source composition and availability in soil and rhizosphere samples, inoculant *Pseudomonas* spp. (shortly pre-starved for C) responded to both source and concentration of C; wheat root exudates gave a response comparable to that of a reducing sugar monomer (glucose), rather than that of common amino acid (glutamate) or carboxylic acid (succinate) components in root exudate¹¹⁷. The cells were later shown to be capable of discriminating the composition of root exudates from plants grown with or without herbicide treatment⁷⁸. Koch *et al.*⁵¹ and van Overbeek *et al.*¹¹¹ used *lacZ*-based C-reporter systems in *P. fluorescens* strains to demonstrate C-limitation in bulk soil, but not rhizosphere. While in general, C may not be limiting in the rhizosphere, the composition and availability of specific organic components can still be important for actual C status of *Pseudomonas*.

The significance of N and P limitation in soil and rhizosphere has also been addressed by studies including bioluminescent *Pseudomonas* reporter strains^{44, 51, 54, 96}. In bulk soil neither N- nor P-limitation could be observed in agreement with the above observations of C-limitation in this habitat^{44, 54}. However, soil amendment with barley straw changed the life conditions for the inoculated *Pseudomonas* strain, which encountered N-limitation as C-rich polymers from the barley residues were degraded^{44, 51}. The rhizosphere (barley) demonstrated significant N limitation, whereas P limitation was not observed^{44, 54}. This work was the first identification of a major nutrient limitation by N of potential significance for growth and activity of pseudomonads in natural rhizosphere. An important recent development is the double reporters, which for example, can address changes in nutrient availabilities in one reporter strain⁵¹. Even the concomitant application of several reporters addressing C, N and P availabilities in the same samples⁹⁶ will probably be useful for future dissections of the nutrient conditions significant to growth and survival of *Pseudomonas* spp. in soil environments.

The N reporter strain used in the work cited above reacted towards limitation by both ammonium and common amino acids (e.g., glutamate), and further work should address if specific N components in exudates may regulate *Pseudomonas* spp. growth in the rhizosphere. Specific reporter bacteria responding to individual amino acids show great promise for identification of such growth-limiting compounds. Induction of a lysine-responsive *P. putida* reporter was demonstrated in rhizosphere (corn), but not in bulk soil³¹ and a tryptophan-reporter strain showed significant induction in older root segments with lateral root formation, but not at the root tip⁴¹. Finally, a *P. fluorescens*

reporter strain was recently used by Kuiper *et al.*⁵⁵ to show that uptake regulation of putrescine, a common polyamine in root exudate (tomato), was important for growth rate and thus competitive colonization ability in the rhizosphere.

Oxygen availability in the soil environment is of fundamental importance to expression of several distinguishing traits in *Pseudomonas* spp., notably denitrification but also a number of redox-regulated traits like fluorescent siderophore and HCN production. High consumption and limited supply rates of oxygen may be expected in rhizosphere, organic aggregates (hot-spots) or highly compacted soil. Nevertheless there is still a poor understanding of the role of oxygen status in soil to diversity of microbial communities, including the selective value of anaerobic traits in *Pseudomonas* spp. In the first attempt to determine oxygen availability by reporter strains, Højberg *et al.*⁴⁰ found induction of a low-oxygen-sensitive *lacZ*-based *P. fluorescens* reporter strain in wetted (85% WHC) but not in unwetted (60% WHC) rhizosphere (barley) and in compacted bulk soil. The work demonstrated that common water and texture conditions easily promoted low-oxygen and thus denitrifying conditions in both rhizosphere and bulk soil. More work based on reporter strains or on knock-out mutant strains is needed to elucidate the role of redox-regulated phenotypes in soil; the latter approach was used by Ghiglione *et al.*³⁶, using a nitrate reductase-deficient mutant of a denitrifying *P. fluorescens* strain and demonstrating that this function may confer a selective advantage in the rhizosphere (corn).

5.3. Molecular Signalling

A fraction (*c.* 40%) of *Pseudomonas* spp. colonizing plant roots was reported by Elasri *et al.*²⁷ to produce N-acyl-L-homoserine lactone (AHL) molecules, serving as bacterial cell-cell communication signals; interestingly, the AHL production appeared to be more common among plant-associated than among soilborne *Pseudomonas* spp. *P. chlororaphis*, *P. fluorescens* and *P. putida* were among the AHL-producers, but may actually produce different AHL molecules. Reporter stains activated by these compounds have further demonstrated that AHL-mediated communication indeed exist among bacteria colonizing tomato roots⁹⁹. In recent years, a *P. aureofaciens* (*P. chlororaphis*) strain 30-84 with AHL-regulated production of extracellular enzymes (protease) and antibiotics (phenazine) has been studied intensively by Pierson and coworkers. Zhang and Pierson¹¹⁸ demonstrated that AHL-regulated gene expression was indeed important for root colonization by this strain.

Specific plant root exudates or fungal signals exerting a direct control on *Pseudomonas* spp. gene expression are currently a popular research topic, both for basic rhizosphere microbiology but only for biotechnology, including the

use of beneficial rhizosphere bacteria for plant protection and growth stimulation. In addition to common root exudate constituents of nutritional value (e.g., carbohydrates like glucose, amino acids like glutamate or carboxylic acids like oxalate), attention has been paid to osmo- or surface-active compounds (e.g., carbohydrates like sucrose and trehalose, amine compounds like betaines and putrescine); the cases of trehalose and putrescine have been mentioned above. A most fascinating observation was that of Steidle *et al.*⁹⁹ reporting that molecular root signals may actually mimic and thus interact with the bacterial (including *Pseudomonas* spp.) cell-cell communication in the rhizosphere¹⁰⁴. Similarly, fungal signals were shown to affect expression of ribosomal (growth-rate determining) RNA genes (*rrn*) in *P. fluorescens*, but the signals have not been identified at a molecular level⁹³.

5.4. Conclusions

During the last decade, marker and reporter gene technologies and advanced microscopy have offered access to single-cell studies of *Pseudomonas* spp. localization, proliferation and metabolic activity in soil microhabitats. Some major conclusions from several independent studies are that growth and metabolism are favored in rhizosphere over bulk soil, however, even on root surface or in rhizosphere only a minority of seed-inoculated *Pseudomonas* spp. cells will remain active over time, typically at discrete microsites along the root. Future studies to improve inoculant efficacy should benefit from these technologies, for example, by further direct observations of both inoculant and indigenous microbial populations during root colonization.

Using *Pseudomonas* reporter strains new knowledge has also been obtained on some important factors in soil or rhizosphere, which limit or stimulate selected strains. It remains a challenge however, to determine whether these observations have a broad validity for other members of the most diverse *Pseudomonas* communities in these environments. Furthermore, new research tasks and information based on reporter bacteria should be targeted towards identification of important selecting factors of *Pseudomonas* spp. diversification in soil and rhizosphere, comparison with the information based on isolation and DNA/RNA-based techniques should thus form the polyphasic approach. Finally, very limited information is yet available concerning reporter-gene expression at the single-cell level and it will be an important future task to obtain information concerning the physiological heterogeneity of *Pseudomonas* spp. populations in these complex natural habitats.

The understanding of AHL-regulated genes, their distribution among *Pseudomonas* spp. and role in soil and rhizosphere processes is far from complete and much is to be awaited from the many studies being performed these years. Generally, it may be foreseen that the “molecular era” of soil and

rhizosphere microbiology has only barely begun, and that the *Pseudomonas* spp. will be important targets for these studies in the future.

In general, the molecular signals representing plant root or fungal stimuli to *Pseudomonas* spp. colonization and metabolism in soil and rhizosphere are yet unknown. A great task in future research is therefore to identify such signals together with their corresponding sensor and signal transduction systems in *Pseudomonas* spp.

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LIFE IN THE RHIZOSPHERE

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1. THE RHIZOSPHERE AND ITS ENVIRONMENT

1.1. General Aspects^{38, 98}

The term rhizosphere is used for the zone of intense bacterial activity around plant roots. It is assumed to extend from the root surface into the soil for up to a few millimeters. The rhizosphere can be divided into the rhizoplane (i.e., the root surface), the endorhizosphere (i.e., the internal root parts) and the ectorhizosphere (in this chapter experimentally defined as the thin layer of sand or soil adhering to the roots). The term rhizosphere was introduced by the German microbiologist Lorenz Hiltner⁷⁶ who described the “rhizosphere effect,” that is, the observation that bacterial levels in the rhizosphere are 10–1000-fold higher than in the surrounding soil. This effect is ascribed to the secretion of nutrients by roots and seeds. These so-called “exudates” attract microbes which feed on exudates. These microbes are often included in a layer of mucigel, a gelatinous material which covers the root. The root cap loses sloughed root cap cells which become heavily colonized and autolyse.

Many plants form mycorrhizal associations with fungi which can function as an extension of the plant root, the “mycorrhizosphere,” and assist the plant in the uptake of water and nutrients⁹⁸.

Plants can have very extended root systems, for example, it has been estimated that one square meter of grassland contains a length of 30 km of root.

The microbial biomass on plant root is limited. The “carrying capacity” is presumably determined by the amount of exuded nutrients. Microbes are not uniformly distributed over the root but restricted to approximately 6% of the root surface^{20, 59, 60, 61, 62, 65, 108, 124} where they form consortia designated as micro-colonies¹³ or biofilms^{37, 94, 116}. The bacteria are usually found at junctions between epithelial cells, indented parts of the epidermal surface or sites of side root appearance^{13, 31}. These areas, together with root tips, are thought to represent regions where exudate is secreted. Root exudates can contain up to 30% of the carbon dioxide fixed by a plant. They contain a large variety of easily digestible components (see Section 1.3).

The rhizosphere contains an extensive micro-flora and -fauna consisting of bacteria, actinomycetes, fungi, protozoa and nematodes. Many physical factors affect the composition of the microbial rhizosphere population, such as soil type, pH, oxygen and water potential. Growing plant roots loosen the soil and transport the microbes they carry⁸⁴.

1.2. Microbes in the Rhizosphere

Exudate attracts many microbes including beneficials, saprophytes and pathogens. Numbers of bacteria, actinomycetes and fungi in rhizosphere soil are estimated to be 10^9 , 5×10^7 and 10^6 per gram, respectively. Scientifically and/or industrially interesting bacteria from the rhizosphere include those forming mycorrhiza, *Azospirillum*, *Bacillus*, *Pseudomonas* and *Rhizobium*. Important fungi include *Alternaria*, *Fusarium*, *Gliocladium*, *Pythium*, *Rhizoctonia*, *Trichoderma* and *Verticillium*.

In the past decades possibilities to follow the fate of microbes in the rhizosphere have enormously increased by the use of genetic markers such as β -galactosidase, ice-nucleation protein and luciferase^{23, 28, 31, 49, 131, 147}. More recently the introduction of autofluorescent protein markers enables their study using CLSM (Confocal Laser Scanning Microscopy)^{13, 14, 145, 150}.

1.3. Root and Seed Exudates

Plant roots release soluble exudate components, mucigel and sloughed off cells. Total rhizodeposition may be a substantial proportion of the plant's photosynthate. Values of 12% to 40% are common. Soluble components will be the best nutrient source for bacteria. Compounds detected in plant root exudates include amino acids, proteins, fatty acids, flavonoids, hormones, organic acids, polysaccharides, organic phosphorous compounds, purines, pyrimidines, sterols, sugars including oligosaccharides, vitamins and unidentified components

Table 1. Estimated concentrations (μM) of identified organic acids, sugars and amino acids in the tomato rhizosphere^a.

Organic acids ^{b,c}		Sugars ^{c,d}		Amino acids ^{e,f,g}	
Citric acid	133	Glucose	20	Glutamic acid	9
Malic acid	57	Xylose	18	Aspartic acid	8
Lactic acid	56	Fructose	7	Leucine	5
Succinic acid	32	Maltose	5	Isoleucine	5
Oxalic acid	31	Sucrose	4	Lysine	4
Pyruvic acid	23	Ribose	2		
Pyroglumatic acid	5				

^aExudate of sterile seedlings of tomato cultivar Carmello was used. The data are based on an estimated tomato rhizosphere volume of 0.5 cm³ per tomato root. The seedlings were incubated in the absence of microbes which, if present, could metabolize exudate compounds but also stimulate exudation.

^bKravchenko *et al.*, unpublished.

^cAnalysed after seedlings were incubated at 20°C for 14 days.

^d96

^eAnalysed after seedlings were incubated at 18°C for 7 days.

^f133

^gArginine plus threonine, asparagine plus serine, glycine plus glutamine, histidine and phenylalanine: All 3 μM or lower.

which inhibit or stimulate fungi, bacteria and nematodes. The amount and composition of exudate is largely affected by multiple factors such as plant species, root region, plant age, pH, temperature and surrounding microbes¹⁰³. Most of the information about root exudates has come from studies of plants growing under sterile conditions in nutrient solution^{121, 124, 139, 151, 152, 153, 154, 155}. The results may therefore not be representative for plants grown under agricultural and horticultural conditions³⁸. In the case of tomato cv. Carmello the major classes of exudate components were identified (Table 1). Consistent with the observation that organic acids are the major class of compounds in tomato exudate, is the observation that *Pseudomonas* mutants unable to utilize sugars are good rhizosphere colonizers⁹⁶ whereas mutants unable to utilize organic acids are not rhizosphere competent⁹⁴.

**2. RHIZOSPHERE COMPETENCE OF
PSEUDOMONAS SSP.**

2.1. Introduction

When an unsterile seed germinates, the microbial inhabitants of the seed coat have excellent opportunities to utilize the seed exudate, colonize the

emerging root and follow it during growth. In soil, they have to compete with the indigenous soil population. Bacteria, which start from the seed and subsequently are able to successfully compete for the growing root tip are designated as rhizosphere competent. Among the most rhizosphere competent bacteria are several *Pseudomonas* ssp.⁹⁴.

In order to identify traits and genes involved in competitive root colonization, the following selection method was developed. Sterile seeds or seedlings are dipped in a 1:1 suspension of two strains and the inoculated seedling is planted in sterile sand moisturized with a plant nutrient solution (Figure 1). After growth for 7–10 days, 1 cm root tip is removed from the plant, bacteria from its rhizoplane and ectorhizosphere bacteria are removed by shaking, and the ratio of the two bacteria is determined. In this way the rhizosphere competence of two bacteria can be compared. It appeared that *P. fluorescens* strains WCS365 and F113 and *P. chlororaphis* strain PCL1391 are among the best competitive colonizers. In order to screen for competitive root colonization mutants, *P. fluorescens* strain WCS365 was mutagenized and the resulting mutant bank was screened for competitive root tip colonization ability⁹⁴. This and other work resulted in the identification of many colonization traits and genes (Table 2).

All colonization-defective mutant derivatives of WCS365 isolated in this way grow as good as the wild type in standard laboratory media. They are not affected in colonization when tested alone, with the exception of auxotrophic¹³⁴ mutants and mutants missing the O-antigen side chain of LPS (lipopolysaccharide)⁴³. After it had been established that they were defective in competitive root tip colonization in the gnotobiotic quartz sand system the mutants were subsequently tested in real soil, in which their colonizing ability also appeared to be impaired in comparison with that of the wild type, except for mutant PCL1217 (de Weert *et al.*, unpublished). It should be noted that real soil contains approximately 10⁸ bacteria per gram, implicating that also in this system competitive colonization is tested.

(Competitive) root colonization can be visualized, using mutant forms of autofluorescent proteins, by CLSM. This technique allows the study of individual cells in the rhizosphere. It enables the study of up to three different populations simultaneously¹⁴.

After seed inoculation and subsequent growth of the plant most bacteria are found on the seed and at the root base and the bacterial density decreases sharply in the direction of the root tip^{31, 78, 88, 93, 134}. Individual bacteria grow out to microcolonies and form biofilms^{13, 31}, usually at junctions between epidermal root cells, indented parts of the epidermal surface and at sites where side roots emerge¹²⁴. Biofilms become covered with mucigel. In lower parts of the root individual cells are visible which follow the growing root, attracted by available exudate nutrients.

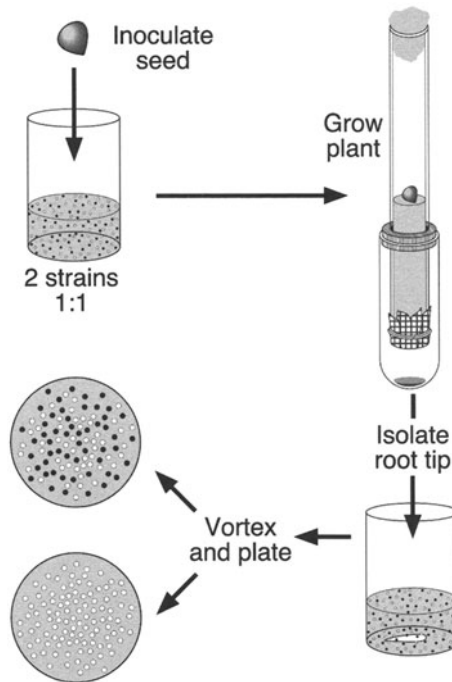


Figure 1. Competitive root colonization assay. A seed or seedling is immersed in a 1 : 1 mixture of two strains and planted in a sterile substrate-plant nutrient solution system. When the root tip is near the bottom of the substrate, after 7–10 days, the plant is taken out and 1 cm of the root tip is shaken in buffer to remove bacteria. The root tip colonizing bacteria are plated on media suited to discriminate the two strains. This method was also used for the screening of competitive root tip colonization mutants.

The microbe-carrying capacity of the rhizosphere is limited. Sterile exudate assumed to more or less mimick rhizosphere conditions allows *Pseudomonas* bacteria to grow to a level of 1 to 3×10^7 cfu/ml⁴³.

2.2. Traits and Genes Involved in Competitive Root Colonization (Table 2)

2.2.1. Chemotaxis. Non-motile and flagella-less mutants loose competition for root tip colonization from their wild type^{44, 50, 134}. Using *cheA* mutants of various *P. fluorescens* strains, de Weert *et al.*⁴⁶ showed that the presence of flagella is not sufficient for competitive colonization but that chemotaxis is also essential. Of the various identified root exudate components tested, sugars did not elicit a chemotactic response whereas amino acids and some organic acids did. Based on concentrations estimated to be present in the rhizosphere, the

Table 2. Functions of *Pseudomonas* genes which are essential for competitive root tip colonization but which are not required for competitive growth in laboratory media.

Classification	Mutant/gene	Impaired function	References
Cell surface structure	PCL 1205; not identified	Synthesis of complete O-antigen of LPS	[43]
	PCL 1208; homologue of <i>rhs</i> element of <i>E. coli</i>	Likely cell surface binding protein	de Weert <i>et al.</i> , unpublished
	PCL 1210; <i>colR/colS</i>	<i>wapQ</i> , encoding a heptose kinase; results in less permeable outer membrane	de Weert <i>et al.</i> , unpublished
Secretion	PCL 1216; homologue of <i>htrB</i>	Transfer of fatty acid to lipidA of LPS	[43]
	PCL 1268; homologue of <i>secB</i>	Chaperone involved in protection of proteins to be secreted	[82]
Bacterial defense	PCL 1206; binding site for regulator protein	Down-regulation of putrescine uptake	[83]
Energy generation	PCL 1085; homologue of <i>maq</i>	Malate dehydrogenase in citric acid cycle	[94]
	PCL 1201; <i>nuo</i> operon	NADH dehydrogenase I; generation of proton motive force	[19]
Specific requirement of building blocks the rhizosphere	PCL 1202; <i>pyrR</i>	Increasing pyrimidine biosynthesis	[18]
	PCL 1218; <i>tyrB</i>	Competitive rhizosphere competence defect can be complemented by uracil	[94]
		Competitive rhizosphere competence defect can be complemented by addition of tyrosine, phenylalanine, aspartic acid and leucine.	
DNA rearrangement	PCL 1233; <i>xerC/sss</i>	Site specific recombinase of the lambda integrase family	[42]
Hypothetical	PCL 1204; homologue of PA 3074 of <i>P. aeruginosa</i>	Unknown	de Weert <i>et al.</i> , unpublished

results indicated that malic acid and citric acid are major chemo-attractants in the tomato rhizosphere⁴⁶.

2.2.2. Type 4 Pili. Camacho Carvajal¹⁸ was able to visualize only one to two type 4 pili per WCS365 cell grown under laboratory conditions. Using mutants in *pilA*, encoding prepilin, and *pilT*, involved in pilus retraction⁷⁴, she showed slight defects in competitive root tip colonization for both mutants. Type 4 pili are expressed in the rhizosphere but attempts to mimick rhizosphere conditions by using exudate did not enhance pilus expression compared to in vitro conditions^{18, 94}.

2.2.3. LPS and Outer Membrane Integrity. Most rhizosphere *Pseudomonas* spp. form a very heterogenous LPS as visualized by extensive ladder patterns⁴⁸. Among competitive root colonization mutants, those lacking the LPS ladder pattern are frequent. These mutants have a decreased growth rate in vitro. However, one competitive colonization mutant with a normal in vitro growth rate and the shorter ladder pattern was found. This indicates that full length O-antigen, although not necessary for optimal growth under laboratory conditions, is required for optimal rhizosphere competence⁴³.

P. fluorescens WCS365 mutant PCL1216 loses competitive root tip colonization and also competition for growth in exudate. It has a mutation in a gene homologous to *htrB*⁴³, encoding a lauroyl transferase that uses (KDO)₂ lipid IVA as the laurate receptor³⁵. Apparently the mutation influences outer membrane integrity⁹⁷ and therefore efficient uptake of nutrients from the rhizosphere.

P. fluorescens mutant PCL1210 is impaired in the two-component regulatory *colR/colS* system⁴⁰. The mutant is slightly resistant towards several antibiotics but more sensitive than the parent towards polymixin B, which removes LPS molecules from the outside of the outer membrane. In addition, the mutant loses in competitive growth from the wild type on a number of carbon sources. These observations point towards an impaired outer membrane permeability. Indeed, permeability of the antibiotic ampicillin is significantly decreased in the mutant (de Weert *et al.*, in preparation).

A mutation in the *orf222* gene of the *orf222-wapQ* operon adjacent to the *colR/colS* operon is also impaired in competitive colonization. The *wapQ* gene, part of the *orf222-wapQ* operon, encodes a heptose kinase. We hypothesize that *colR/colS* is required for expression of *wapQ* and that this activity is required to fully open the protein pores in the outer membrane in order to allow the cell to compete optimally for nutrients in the rhizosphere (de Weert *et al.*, in preparation).

2.2.4. Ability to Synthesize Vitamins and Building Blocks for Macromolecules. Amino acid auxotrophic mutants of *P. fluorescens* WCS365 are impaired in

potato root colonization in field soil⁶⁸. Consistent with this observation several amino acid auxotrophs of *P. fluorescens* WCS365 were found among competitive colonization mutants. Even when tested alone, these mutants were unable to colonize the root tip. Colonization was restored by the addition of the appropriate amino acid¹³³.

Mutant PCL1202 of *P. fluorescens* WCS365 has a mutation in *pyrR*. Addition of exogenous uracil restores the defects of the mutant with respect to competitive growth on exudate and competitive tomato root tip colonization. Expression studies indicated that PyrR positively affects transcription of the *pyrB* promoter, suggesting that enhanced pyrimidine synthesis is required for competition in the rhizosphere¹⁸.

A vitamin B₁ auxotroph of *P. fluorescens* WCS365 appeared to be a competitive tomato root tip colonizer¹³³. A similar observation was reported for a biotin auxotrophic *Rhizobium meliloti* mutant in the alfalfa rhizosphere¹³⁹. Apparently concentrations of vitamins present in exudate are not sufficient to allow growth of these vitamin auxotrophs.

2.2.5. Utilization of Exudate Components. The major classes of soluble tomato root exudate components in increasing order are amino acids, sugars and organic acids (Table 1). We analysed to what extent utilization of exudate components affected rhizosphere competence. It appeared that *P. fluorescens* WCS365 mutant PCL1083 with a mutation in *zwf*, which encodes glucose-6-phosphate dehydrogenase, is impaired in growth on the sugars glucose, fructose, sucrose and xylose but grows normally on organic acids. The mutant is not impaired in competitive tomato root tip colonization⁹⁶. In contrast, mutant PCL1085, which grows poorly on the organic acids malic acid and succinic acid and is affected in the expression of the *mgo* gene encoding malate dehydrogenase, is a poor competitive tomato root tip colonizer. The results show that a mutant unable to use the major exudate components is a poor competitive colonizer and indicate that efficient utilization of major exudate carbon sources is the nutritional basis for competitive root tip colonization. Support for the notion that utilization of major exudate compounds is crucial for efficient growth in and colonization of the rhizosphere was obtained from work with *P. putida* strain PCL1444. This strain was selected as an efficient grass root tip colonizer⁸⁴ and growth rate on the major grass root exudate components succinic acid, glucose and citric acid is very high. This result suggests that selection for efficient competitive root tip colonization selects for efficient growth on major root exudate compounds⁸⁵.

2.2.6. Protection Against Toxins in Exudate. Uptake of polyamine putrescine is limited to a certain level. In *P. fluorescens* mutant PCL1206 the binding site of the regulator protein gene upstream of the *pot* operon is supposed to be

mutated, resulting in excessive putrescine uptake and transient bacteriostasis. The results obtained with this mutant suggested that putrescine is present in tomato root exudate. This was experimentally confirmed. The results show that *Pseudomonas* protects itself against high putrescine levels in the rhizosphere⁸³.

P. putida mutants impaired in seed colonization have been identified by Espinosa-Urgel *et al.*⁵⁷. Of the eight mutations only three were similar to known genes. Of these, a potential multi-drug efflux pump could be involved in protection against toxins in exudate.

2.2.7. Phase Variation. One of the most interesting competitive root tip colonization mutants of *P. fluorescens* WCS365 is mutant strain PCL1233. In this strain the crucial gene affecting colonization is a *xerC/sss* homologue⁴². Sss in *P. aeruginosa* is a member of a family of site-specific recombinases which play a role in phase variation through conservative reciprocal recombination between two homologous DNA fragments approximately 15-bp in length. Depending on the orientation of these fragments Sss activity results in inversion or excision of the DNA fragment between the recognition sites¹²⁶.

Phase variation regulates phase-variable surface antigens. Phase variation helps pathogenic bacteria to escape the animal's defense system⁵⁶. From the work of Dekker *et al.*⁴² it appears that Sss also regulates competitive colonization. Apparently mutant PCL1333 is frozen in a DNA orientation which is unfavourable for rhizosphere competence.

The role of Sss in rhizosphere colonization was confirmed by Sanchez-Contreras *et al.*¹²⁷ who reported that *P. fluorescens* F113 undergoes colony phase variation in the alfalfa rhizosphere. One of the variants, called F, showed alterations in siderophore, cyanide and exoprotease production. This phenotype was selected in the rhizosphere and could be complemented by *gacA*. Mutation of *sss* showed that most of the phenotypic variation which occurs during rhizosphere colonization was the result of Sss activity.

Chabeaud *et al.*²² found that *P. brassicacearum* strain NFM421 can form two colony types designated as phases I and II. Phase II cells appear at the edges of small mucoid phase I colonies as large, flat colonies which neither express fluorescent pigmentation, indicative for siderophore production, nor protease and lipase.

Van den Broek *et al.*¹⁵⁶ studied 43 antagonistic, phase-variable, *Pseudomonas* isolates from maize roots which form opaque phase I and translucent phase II colonies. It appeared that important biocontrol traits, such as motility and the production of anti-fungal metabolites, protease, lipase, chitinase and biosurfactants are correlated with only phase I colony morphology. Two mutants of the selected *Pseudomonas* strain PCL1171 with stable phase II expression were mutated in *gacS* whereas a third mutant with

increased colony phase variation frequency has a mutation in *mutS*. Only phase I cells resulted in efficient suppression of take-all disease of wheat caused by the fungus *Gaeumannomyces graminis* var. *tritici*. Neither the *gacS* mutants nor the *mutS* mutant was able to suppress take-all, but biocontrol activity was restored after genetic complementation of these mutations. Mutation in a gene encoding a lipopeptide synthase homologue had lost all antagonistic and biocontrol ability, indicating that biocontrol by strain PCL1171 is dependent on the production of a lipopeptide. These results show that the phase variation process is dependent on *gacS* whereas *mutS* is probably involved in repairing *gacS* mutations. It can be concluded that the two major biocontrol traits, competitive root tip colonization²⁹ and antifungal metabolite production^{30, 142, 156} can be subject to phase variation. Therefore, phase variation not only plays a role in escaping animal defense but plays a much broader role in the ecology of bacteria producing exo-enzymes, antibiotics and other secondary metabolites¹⁵⁶.

2.2.8. Other Colonization Mutants. Two NADH dehydrogenases have been identified in *P. fluorescens* WCS365¹⁹. Of these, NADH dehydrogenase I is encoded by a 14-gene *nuo* operon whereas NADH dehydrogenase II is encoded by the *ndh* gene. Mutation of *nuo* but not of *ndh* affects competitive tomato root tip colonization negatively^{19, 43}. NADH dehydrogenase I is involved in the generation of the proton motive force which can be used for the uptake of various nutrients, generation of ATP and ATP-dependent rotation of flagella. Since efficient nutrient uptake (Section 2.2.3) and chemotaxis (Section 2.2.1) are very important processes for rhizosphere competence, it is clear why expression of *nuo* in the rhizosphere is important.

Mutant PCL1268 is impaired in *secB* and loses competitive colonization from its parent WCS365. SecB acts in *Escherichia coli* as a chaperone involved in export of proteins over the cytoplasmic membrane. We assume that, since no secreted proteins are known for WCS365, *secB* is involved in the secretion of periplasmic or outer membrane proteins which play a role in colonization^{82,94}.

2.2.9. New Gene Functions Revealed by Studying the Life Style of *Pseudomonas* in the Rhizosphere. In the past century the genetics behind bacterial traits was mainly studied under laboratory conditions. Identified gene functions therefore relate to those conditions. The challenge of the 21st century is to study bacterial life styles in their natural environments. In our search for genes specifically involved in rhizosphere colonization we selected only those genes which grew well under laboratory conditions (Section 2.1). Therefore it is not surprising to find among colonization genes many genes of which the function has not been identified yet (Section 2.2 and Table 2). Similar results

were found for genes expressed in the rhizosphere but not in laboratory medium¹²³ and for genes playing a role in adhesion to seeds⁵⁷. We conclude that studies on genes expressed exclusively in interactions with the plant will identify a role for many of the so far not identified *Pseudomonas* genes.

3. COPING WITH NEIGHBOURS IN THE RHIZOSPHERE

Some *Pseudomonas* strains act as *biofertilizers* by converting atmospheric nitrogen into ammonia which can be used by the plant as a nitrogen source¹³⁷. Other biofertilizers solubilize sparingly soluble inorganic phosphate or degrade organic phosphate into soluble phosphate¹⁴⁶. *Phytostimulators* are bacteria which stimulate germination or plant growth in the absence of pathogens. Pseudomonads are known to cause such effects through the production of auxins⁸¹ or ethylene^{69, 159}. *Rhizoremediators* are rhizosphere-competent bacteria which detoxify one or more xenobiotics in the rhizosphere. A good example is *Pseudomonas putida* strain PCL1444 which was selected from poly-aromatic-hydrocarbon polluted rhizosphere soil for good colonization as well as for the ability to degrade naphthalene⁸⁴. *Mycorrhization helper bacteria* include pseudomonads which enhance mycorrhization of plant roots and therefore the plant's ability to take up water and nutrients from its surrounding^{55, 64, 67}. *Biocontrol agents* are bacteria which protect plants against diseases. *Pseudomonas* biocontrol agents will be treated in section 6. It should be noted that also the plant genotype can be an important factor in biocontrol¹³².

Because interactions in the rhizosphere are often referred to as "microbe-plant interactions," interactions between different microbes in the rhizosphere are often underestimated. Microscopical and molecular analyses of *Pseudomonas*-fungus interactions have shown a number of interesting phenomena. (a) Direct physical interaction between pseudomonads and fungi have been well-documented^{11, 16} whereas *P. fluorescens* WCS365 adhered well to, and can form a coat around, the mycelium of the arbuscular mycorrhizal fungus *Glomus intraradices*^{11, 118}. *P. fluorescens* CHAO cells did not but its mucoid mutant adhered well¹⁰. *P. fluorescens* F113 stimulates mycelium development of *Glomus mosseae* spores as well as tomato root colonization⁷. (b) Spores of the endosymbiont *Gigaspora margarita* contain bacteria of the species *Burkholderia* whose function has not been established yet¹¹⁸. (c) *P. stutzeri* isolates were described to attach to chlamydospores and also to decrease the germination of *Fusarium* chlamydospores¹⁴⁷. (d) The microbial partners involved in biocontrol of tomato foot and root rot actively attack each other. (e) *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391 colonize

hyphae of the pathogenic fungus F.o.r.l. (*Fusarium oxysporum* f.sp. *radicis-lycopersici*) in the tomato rhizosphere to such an extent that the resulting bacterial biofilm may disable the fungus. The production of PCN by PCL1391 affects hyphal growth and branching and also redirects hyphal growth¹⁶. Investigation of whether the bacterium *P. fluorescens* WCS365 is attracted to the fungus showed active chemotaxis towards F.o.r.l. The major chemoattractant was identified as fusaric acid. Co-cultivation of WCS365 and F.o.r.l. resulted in colonization of hyphae (de Weert *et al.*, in preparation). The fungus also defends itself against some biocontrol agents: Synthesis of the anti-fungal metabolites Phl¹¹² and PCN (van Rij *et al.*, unpublished) is negatively affected by the presence of the F.o.r.l. product fusaric acid. (f) Signals of *Pythium ultimum* repress expression of two ribosomal RNA operons of *P. fluorescens* F113. Mutants in these genes are reduced in rhizosphere competence^{58, 136}.

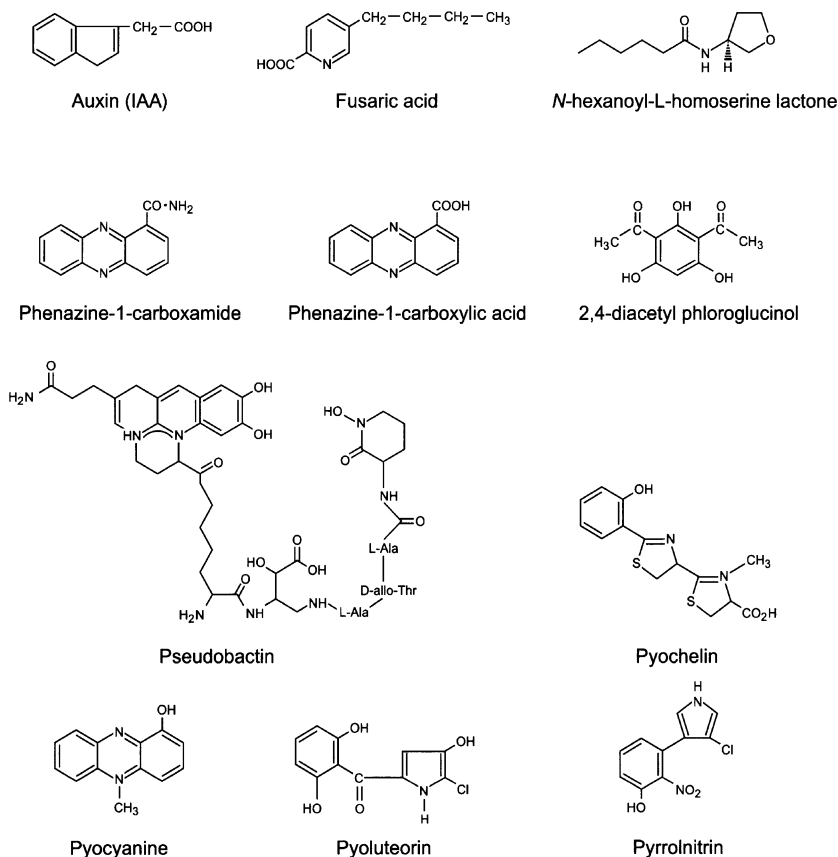


Figure 2. Structures of a number of secondary metabolites produced in the rhizosphere.

(g) Upon contact with the fungus *Phytophthora*, five genes are induced in *P. putida* which include genes showing high homology with diacyl glycerol kinase, ABC transporters and outer membrane porins⁹¹.

N-acylhomoserine lactone (AHL; Figure 2)-mediated quorum sensing by bacteria regulates traits that are involved in pathogenic, symbiotic and surface-related relationships between microbial populations and between microbial populations and their host^{8, 25, 39, 66, 72, 163}. These interactions have also been established in the rhizosphere^{9, 92, 119, 141}. Conjugation requires AHLs¹²⁰. Therefore it is likely that the high frequency of gene transfer in the rhizosphere^{148, 157} is the result of the high local bacterial density in the rhizosphere³¹. Plants can secrete substances which mimic the activities of AHLs¹⁴¹. Transgenic plants have been constructed which secrete AHLs^{63, 99}. *Bacillus* bacteria can produce a lactonase enzyme activity which inactivates AHLs. Incorporated into *Erwinia*, it attenuates its virulence⁵¹.

4. PSEUDOMONAS BIOCONTROL AGENTS

4.1. Introduction

Up to 30% of the world's crop plant yield is lost due to diseases and pests. Many chemical pesticides are detrimental to human health. Use of resistant plants is a good alternative but when these are genetically modified their use is not always accepted by the public. This is especially the case in Europe. An attractive and more flexible alternative is the use of biological control agents. In the context of pseudomonads, these are strains which reduce diseases of plants which usually are caused by pathogenic fungi. In the rhizosphere these agents act against soil-borne pathogens such as *Fusarium*, *Gaeumannomyces*, *Rhizoctonia* and *Rossellinia*.

Biocontrol is studied in a tripartite system consisting of plant, pathogen and biocontrol microbe. Also the substrate in which the plant grows (e.g., soil, stonewool) plays an important role. Biocontrol agents can be obtained by direct screening of microbes or after pre-screening of microbes for AFM (anti-fungal metabolite) production and/or excellent root tip colonization after inoculation of the seed (Figure 3).

In the past, attack of plant roots by pathogens has been visualized by classical electron microscopy¹²⁴. The recently developed possibility to mark organisms with differentially labelled autofluorescent proteins has enabled us to study, in a noninvasive way and using a confocal laser scanning microscope, (a) establishment, colonization and penetration of roots by the fungal pathogen⁸⁷, (b) colonization of the root by biocontrol bacteria^{12, 13, 14, 145} and (c) the effects of *Pseudomonas* biocontrol agents on this process^{16, 30}.

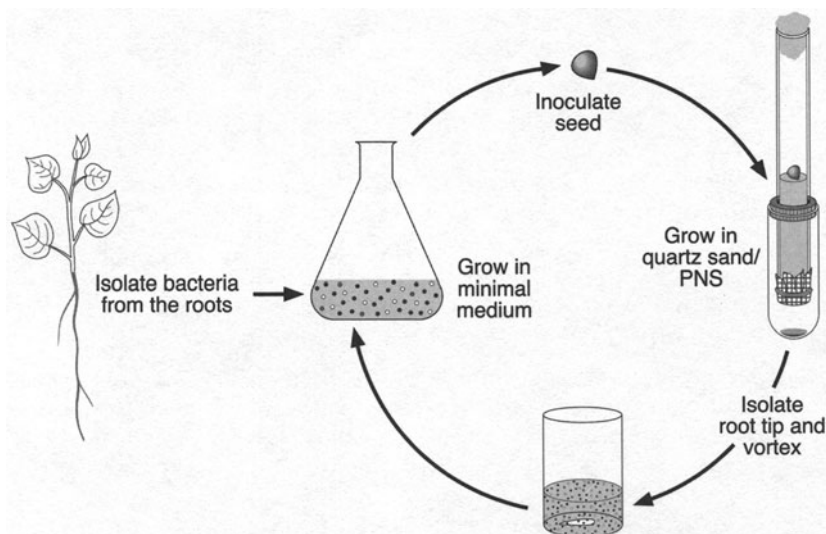


Figure 3. Procedure for the enrichment of enhanced competitive root tip colonizers. Rhizosphere bacteria are shaken from suitable plant roots and used to inoculate a seed. After growth of the plant, the bacteria are shaken from the root tip and used to inoculate a second seed. Those bacteria which are still able to colonize the root tip after 3 cycles are screened for enhanced colonization (After ref. 84).

4.2. Mechanisms used by *Pseudomonas* Biocontrol Agents³³

4.2.1. Antibiosis. Strains acting through antibiosis are usually identified by testing them for antagonistic activity on plates on which the target pathogen is also inoculated. Between 1%³¹ and 21%¹⁵⁶ of rhizosphere pseudomonads produce AFMs, depending on the source and possibly on the history of the field of origin¹⁵⁶. The most frequently found AFMs are 2,4 diacetylphloroglucinol (Phl)^{6, 44} and phenazines, such as phenazine-1-carboxylic acid (PCA; ref. [142] and phenazine-1-carboxamide acid (PCN; ref. [27]) or oxychlororaphin⁴. Structures of these and other AFMs are shown in Figure 2. Single *Pseudomonas* strains can produce a complex mixture of secondary metabolites, including hydrogen cyanide, siderophores, such as pyoverdine and pyochelin, and AFMs such as Phl, PCA, PCN, pyoluteorin and pyrrolnitrin^{52, 115, 142} (Figure 2). Especially *P. fluorescens* strains CHAO^{72, 89} and Pf-5¹¹³ produce complex cocktails of secondary metabolites. Tomashow and Weller¹⁴² have written an excellent review on the role of AFMs in biological disease control.

Recently it has been found that several biosurfactants of the class of cyclic lipopeptides have antibiotic properties towards root-pathogen

fungi^{79, 86, 106, 110, 138, 143}. Although it was thought initially that they act by their biosurfactant activity towards zoospores¹³⁸ it is now clear that they also have other antibiotic activities since they can also antagonize non-zoospore forming fungi¹¹⁰. A frequency as high as 60% of the *Pseudomonas* strains isolated from a sandy soil in Denmark produce cyclic lipopeptides¹¹⁰. Cyclic lipopeptides with antibiotic activity include amphisin⁷⁹, tensin¹¹¹ and visconamide¹⁰⁹. Synthesis of amphisin⁷⁹ as well as that of the biosurfactant produced by *Pseudomonas* PCL1171 (156; see Section 2.2.7) is dependent on a *gacS* homologue. In the first case the synthesis is triggered by sugar beet exudate⁷⁹.

Antagonistic activity by no means guarantees biocontrol activity. In order to act as a biocontrol agent, a strain should produce the AFM under rhizosphere conditions at the right time and deliver it over those parts of the root system which are vulnerable for attack by the pathogen. The following findings explain why antagonistic activity in vitro is not sufficient for biocontrol activity. (a) In the case of *P. chlororaphis* it has been proven that efficient root colonization is a prerequisite for biocontrol²⁹. It is likely that this is the case for all biocontrol strains which act through antibiosis. (b) The syntheses of AFMs are extremely sensitive to environmental conditions such as carbon and nitrogen source, concentrations of amino acids, cations, anions and oxygen, of pH and of salt stress ref. [27], [32], [53], [130], van Rij *et al.*, unpublished). All these factors can play a role in the rhizosphere, where they can interplay with the complexly regulated synthesis of secondary metabolites in the bacterial cell in which the *gacA/gacS*, *rpoS* and several events at the post-transcriptional level play a role^{1, 2, 15, 72, 73, 129, 161, 162}. (c) Instability of AFM production can also be a problem. Mutations in the *gacA/gacS* system, which controls AFM production, occur at a high rate and during production of biocontrol agents up to 61% unproductive *gac* mutants have been found⁵⁴. In the rhizosphere, a mixture of wild type and *gac* mutants seems to be beneficial for the survival of wild-type populations²⁴. Mutation of the *gac* system can sometimes be visible as alteration in colony morphology^{54, 127, 156}. Instability of *gac* genes can be limited in several strains by mineral amendments or by diluting media⁵⁴. (d) Suppression of AFM synthesis by metabolites^{112, 129} and exo-enzymes¹⁵⁹ of other rhizosphere organisms.

4.2.2. Competition for Nutrients and Niches. All living organisms require divalent iron ions for growth. Iron limitation occurs because of its low solubility at neutral or alkaline conditions. Many bacteria produce siderophores upon iron ion limitation. These are small molecules with an extremely high affinity for ferric iron ions. The siderophore-Fe³⁺ complex is taken up by cells by specific outer membrane receptor proteins. Internally Fe³⁺ is converted into Fe²⁺. Siderophores produced by *Pseudomonas* ssp. include pyochelin, pyoverdine, pseudobactin and ferribactin. Some biocontrol agents produce

large amounts of effective siderophores. Intriguing is that a strain such as *P. putida* WCS358 is able to take up a range of different siderophore-Fe³⁺ complexes^{80, 122}. In conclusion, competition for ferric iron ions forms a well-documented example of competition of biocontrol bacteria with pathogenic fungi for nutrients.

Bolwerk *et al.*¹⁶ clearly showed that F.o.r.l. and *Pseudomonas* biocontrol strains WCS365 and PCL1391 compete for the same sites on the root. Bacteria applied on seeds reached the root surface faster than F.o.r.l. applied as spores in the substrate. The presence of the biocontrol agents diminishes the biomass of the fungus on the root substantially. Since bacteria are attracted by seed exudate components (deWeert *et al.*, in preparation) this form of competition presumably represents competition for sites as well as competition for nutrients.

4.2.3. Colonization of Fungal Hyphae. The biocontrol pseudomonads WCS365 and PCL1391 not only colonize the tomato root, they also colonize F.o.r.l. hyphae extensively¹⁶. It has been hypothesized that this process contributes to biocontrol by preventing the fungus from attacking the root^{16, 95}. It is likely that chemotaxis towards fusaric acid (Figure 2) is the step prior to hyphae colonisation (deWeert *et al.*, in preparation).

4.2.4. Induced Systemic Resistance (ISR). Some *Pseudomonas* bacteria have the ability to induce a state of systemic resistance in plants, which provides protection against a broad range of phytopathogenic organisms including viruses, bacteria and fungi¹⁵⁸. Bacterial determinants which induce ISR include structurally widely different factors such as flagella, LPS, salicylic acid and a combination of the siderophores pyochelin and pyocyanin^{3, 45, 158}. The phenomenon resembles innate immunity in mammals¹⁴⁹ in which flagellin is recognized by Toll-like receptor-5 and activates a systemic defense response^{75, 135}. Since innate immunity also occurs in plants and since the systems might have similar mode of actions^{70, 114}, we hypothesise that the different ISR inducing molecules activate the innate immune systems through different Toll-like receptors. Such a mechanism would allow the plant to sense, and protect itself against a large group of different microbes.

4.3. Enhancing Biocontrol

Presently the efficacy of biocontrol agents is not optimal. Two biological factors have been shown to be able to improve the robustness of strains, namely competitive colonizing ability and level and diversity of AFM production in the rhizosphere^{17, 160}. A third important practical factor is the period of time that microbes can be stored ("shelf life") before they are applied.

Three ways are known to improve colonization. First, by providing more or less unique nutrients to the microbe to be favoured. For example, plants have been engineered to produce large amounts of opines and beneficial microbes can be engineered to utilize these opines¹²⁸. Another example is the amendment of salicylic acid to promote salicylate-utilizing pseudomonads³⁶. Similarly, *P. fluorescens* WCS307, a poor tomato root colonizer, can become dominant in the rhizosphere when maltose is added. This strain grows better on maltose than several other pseudomonads⁹⁶ and apparently also better than many indigenous microbes. A second way to improve competitive root tip colonization is by introducing multiple copies of the *sss* gene⁴¹, encoding a site-specific recombinase involved in phase variation, in wild-type *P. fluorescens* strains WCS307 and F113⁴¹. Apparently the percentage of rhizosphere competent cells in their populations is thereby increased. A third strategy to isolate enhanced colonizers is the use of the enrichment procedure described by Kuiper *et al.* (ref. [84]; Figure 3). Starting with crude rhizosphere microflora, those bacteria which are dominant colonisers can be enriched. The procedure even results in biocontrol strains (F.D. Kamilova, unpublished). Using the same enrichment technology on Tn5 mutants, one mutant was found which contains a Tn5 mutation in the mutator gene *mutY*. Control experiments showed that mutation of *mutY* alone severely impairs colonization. Apparently the enhanced colonizing strain has collected a combination of mutations beneficial for effective colonization in the *mutY* background (de Weert *et al.*, unpublished).

AFM production can be increased by decreasing the frequency of *gac* mutations, by growth in the presence of certain minerals or by growth in diluted medium⁵³. Also, AFM biosynthetic genes can be expressed under a strong promoter¹⁰⁵, the spectrum of AFMs produced by a bacterium can be broadened^{5, 144} or the introduction of a AFM biosynthetic gene can extend the host range of a biocontrol strain³⁴.

Pseudomonas bacteria do not form endospores. Consequently they cannot be stored for a long time. Formulation, for example, incorporation of bacterial cells in a carrier such as alginate¹²⁵, increases their life time and therefore improves chances of application.

4.4. Evolution of Rhizosphere Competence and other Biocontrol Traits

Many of the genes involved in competitive root tip colonization also appear to play a role in the colonization of animal and human tissues and/or appear to function as virulence factors (ref. [140]; Table 3). This suggests that colonization of eukaryotic tissues by bacteria occurred early in evolution. It is likely that the same applies for other biocontrol traits. For example, phenazine

Table 3. Competitive *Pseudomonas* plant root tip colonization genes and traits which also play a role in colonization of animal and/or human tissues.

Genes/traits	Role in interaction with animal/human tissue	References
Motility, flagella	Virulence factor in burn wound sepsis by <i>P. aeruginosa</i>	[21]
Motility	Colonization of host light organ by <i>Vibrio fischeri</i>	[71]
LPS	Colonization of animal tissues	[26], [100], [102], [107], [117]
LPS (<i>htrB</i>)	by <i>Salmonella typhimurium</i> , <i>V. cholerae</i> , <i>K. pneumoniae</i> , <i>E. coli</i> Colonization of organs of lymphatic system of mouse by <i>S. typhimurium</i> , <i>V. cholerae</i> , <i>Klebsiella pneumoniae</i> , <i>E. coli</i>	[77], [90]
Auxotrophic genes, biotin synthesis and pilus genes	Colonization of mouse intestine and GI tract	[26], [102]
Many (novel) factors	Colonization of infant mouse small intestine by <i>V. cholerae</i>	[104]
	Human intestinal cells	[101]

derivatives do not only kill fungi, but the phenazine-derivative pyocyanine, produced by *P. aeruginosa*, is also involved in killing animal cells and tissues¹⁰⁰.

5. FUTURE PROSPECTS

Most work on the analyses of exudates was done in the period 1950 to 1980. Recently developed sensitive analysis and high throughput techniques should be a stimulus to investigate in more detail which compounds are present in root and seed exudates ("rhizosphere metabolomics"). Also, the dynamics of exudate in relation to biotic and abiotic factors should be investigated.

Root and seed colonization are very complex processes in which many genes and traits are involved, including those which might be unique for life in the rhizosphere. By using transcriptomics, one should be able to identify genes which are specifically expressed in spermosphere and rhizosphere as a result of interaction with the plant or with other organisms. Subsequent mutation analysis should reveal new information on life in the rhizosphere as well as on genes which are crucial for rhizosphere colonization and biocontrol. Vice versa, the influence of rhizobacteria on plant gene expression should be investigated using transcriptomics.

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LIFE IN THE PHYLLOSPHERE

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1. INTRODUCTION

The microbiology of the aerial portion of plants supports a diversity of microorganisms, including bacteria, fungi and archaeae. In addition there are direct interactions with eukaryotic species that feed off the plant. The abundance of life in the phyllosphere is matched by the habitat range that plants occupy in both terrestrial and aquatic environments. Plant leaves provide the greatest surface area on the planet tolerating geographic and climatic extremes that can fluctuate on a daily cycle from sub-zero night time temperatures to leaf surface temperatures that exceed 50°C in direct sunlight. Plants are found in over 90% of the approximately 2×10^8 km² terrestrial surface of the planet where the surface area of leaves (the phylloplane) approaches 1×10^9 km² [ref. 75]. It is a statement of fact that those bacteria that have adapted to life in the phyllosphere must exhibit a range of phenotypic characteristics to mitigate against the effect of these physical parameters. These are perhaps greater than those experienced by soil or rhizosphere bacteria. However resource limitation, in respect to nutrient supply and water availability are common selective factors that dictate the range and functional capacity of microbial life at plant surfaces. To understand more about the wider context of leaf microbiology the

reader is directed to the conference proceedings of the two most recent symposia on phyllosphere microbiology^{66, 78} and recent reviews that cover a number of related issues that have not been dealt with in depth here^{64, 65}.

In this chapter the diversity and ecology of pseudomonads will be considered although examples from other groups will be taken for comparison. Bacteria remain the focus of phyllosphere study as they are reported to be the numerically most abundant of isolates recorded using both isolation and culture independent methods. These general observations however, are not intended to imply that yeast and other fungi have a less important role; indeed some estimates of the population density of yeast phylloplane communities indicate that representatives of the sporobolomycetes in certain plants may represent the greater microbial biomass in older leaves⁷⁸. Appropriately pseudomonads have received the most attention, due in part to their relative abundance and because they are amenable to genetic manipulation. These latter attributes have allowed considerable insight to the ecology of a select number of species and contributed to a more detailed functional understanding of the population genetics and genetic diversity of leaf bacteria needed to identify the key adaptive traits they possess for life in the phyllosphere.

2. DISTRIBUTION, NUTRIENT AVAILABILITY AND LIMITATIONS

The phyllosphere is an exposed habitat prone to physical and chemical extremes that impose considerable selective pressures on resident communities, resulting in fluctuations in both the diversity and density of microbial populations. This variable and unbuffered nature makes the study of plant surface microbial ecology particularly challenging. Not least as airborne and water splash deposition of microorganisms on plant surfaces confuses the differentiation between true phyllosphere colonists and airborne surface contaminants. Nutrient and water availability can be limiting although there are accessible, but limiting amounts of amino acids, carbohydrates or organic acids are freely available at the leaf surface. The work of Lindow and co-workers has provided a unique insight to bacterial distribution and nutrient availability at the phylloplane through the use of specific biosensors. These studies demonstrate that simple sugars including sucrose, glucose and fructose leach from the leaf and constitute the dominant carbon source available⁷². The use of *Erwinia herbicola* based sensors that carry *gfp* or *inaZ* reporter genes fused to the *scrY* sucrose and fructose induced promoter, or *fruB* (fructose responsive promoter) revealed spatial heterogeneity of nutrient distribution detected in situ on bean leaves using these bacterial sensors^{51, 73}. Surprisingly in

another study using a *Pseudomonas syringae* Fe⁺³ sensor the authors concluded that the majority of cells on leaves do not experience iron limitation³⁷. These data reveal localisation relative to nutrient availability which may explain the “patchy” distribution of colonies or aggregates of bacterial epiphytes on the leaf surface and the apparent poor performance and survival of arriving immigrants⁶⁴. Additional nutrients can diffuse via the waxy cuticle, particularly after it has been damaged by infection or aging. Temperature, desiccation, oxidative and osmotic stresses, infrared and ultraviolet (UV) radiation can fluctuate diurnally, particularly on the upper (adaxial) surface of the leaf⁶⁵. These extremes stress bacterial communities, particularly UV which acts as a bactericidal agent as well as being mutagenic. Organisms may avoid infrared and UV light by persisting on the lower (abaxial) surface of the leaf or by the expression of genetic resistance determinants such as *rulAB*^{32, 105}. Water availability is perhaps one of the limiting factors to successful phylloplane survival. Free water arrives at the leaves in the form of rain, dew, fogs and irrigation. It has been estimated that, 30 mg cm⁻² water can be retained, equivalent to rainfall of <2 mm. The water holding capacity will vary according to leaf surface properties (topography, hair like structures, waxy cuticle etc.) and general architecture of the aerial parts⁷¹. In many plants, leaves are orientated towards the base of the stem which directs water to the growing apex and roots. Furthermore, EPS production is common in many bacteria and not only buffers collections of cells from water, pH and oxidative related stresses but these extracellular structures can also act as a sink for the concentration and collection of nutrients released by the plant^{44, 65}. In addition, as general observation, bacterial colonies isolated from leaves tend to be highly pigmented, this is less common in soil and rhizosphere bacteria.

3. BIOFILMS, AGGREGATES AND CELL SIGNALING

As explained above and in the detailed studies of Morris *et al.*^{76, 77} bacteria do appear to form small biofilms on leaves, particularly young and emerging leaves where humidity and nutrient availability may be high. The formation and maintenance of biofilms is considered to be a function of community or multicellular coordination^{17, 86} mediated by density dependent quorum sensing which are often associated with the ability to produce and respond to signaling molecules defined by homoserine lactones. To demonstrate their role transgenic tobacco plants were constructed to express N-acyl-homoserine lactone (AHL), which coordinates the expression of genes encoding the main virulence determinants of the soft-rot phytopathogen *E. carotovora*.

These transgenic plants had a significantly reduced hypersensitivity response than wild-type plants due to the reduced bacterial antagonism⁶⁸. This approach not only provides a clear indication of the importance of quorum sensing but also demonstrates the potential for GMOs in combating bacterial pathogens and spoilage organisms that result in serious agronomic loss. In related phytosphere studies by Molina *et al.*⁷⁴ the lactonase gene (*aiiA*) from *Bacillus* A24, which degrades AHLs, was introduced into *P. fluorescens* P3. When inoculated in planta the transgenic, lactonase producing pseudomonad colonised the phytosphere and suppressed soft rot of potato and crown gall in tomato which are caused by *E. carotovora* and *Agrobacterium tumefaciens* respectively. Quite how relevant AHL production and related factors are in cell signaling and density dependent sensing in the phyllosphere microbiology of epiphytes has yet to be determined. However, a number of secondary metabolites, such as antifungal compounds, are regulated by *luxI*R homologs and AHLs have been extracted from the phytosphere of certain plants^{86, 87}. As indicated above *Erwinia* spp. and other plant pathogens including *Ralstonia solonacearum* have complex quorum signaling pathways that regulate the density dependent expression of their virulence factors. Clearly leaf colonists are receptive to and sense their surroundings which may in part explain the apparent distribution of bacteria in aggregates in preference to their existence as isolated single cells. Perhaps the best example of the importance of AHLs in the colonisation of the phylloplane can be drawn from the elegant work conducted in the marine environment. Givskov *et al.*²⁵ demonstrated that the micro alga *Delisea pulchra* produces structural homologues of AHLs which inhibit AHL controlled processes in bacteria. These compounds in effect prevent colonisation and hence biofilm formation. Such biofouling can be severe on marine algae and leads to tissue damage and loss of structural integrity. The seaweed *D. pulchra* has surface associated specialised cells that produce these halogenated furanones⁶⁹ as a defense mechanism against biofouling. Furanone production affects not only the abundance and distribution of bacteria on its surface but also the community composition in that high proportions of Gram-positive bacteria were found, which is in contrast to the predominance of Gram-negative bacteria typical of marine phyllosphere systems⁴³.

4. PHYLLOSPHERE MICROBIOLOGY

The surface and interior of aerial parts of plants, including flowers, fruits, stems and leaves represent the phyllosphere. Specialised microbial colonists, phytopathogens, spoilage organisms and periodic immigrants have all been described for this diverse habitat. As plants represent one of the most

important features of our landscape and our primary food source it is somewhat surprising that only a limited number of detailed investigations have been conducted that describe their above ground microbiology. This is in stark contrast to the attention that soil rhizosphere systems receive. Pertinent investigations reveal a diverse and specialised microbial community that is distinct from the rhizosphere. Bacteria are by far the most numerically abundant colonisers, typical community densities in the order of 2×10^7 cells/cm⁻² of leaf surface have been recorded by traditional methods¹, although this can vary from 10s to in excess of 1×10^{12} g⁻¹ in arid and senescing leaves respectively. Densities vary over the plant surface, and in leaf buds or on young emerging leaves densities of bacteria may be two orders of magnitude greater than in established mature leaves. These differences can in part be explained by the increased availability of water as these regions are often protected or collect moisture derived from rainfall or dew. In addition nutrient loss from young, developing leaves provides suitable C and N source for colonising bacteria which typically form complex mixed assemblages or biofilms that facilitate survival. These assemblages also represent suites of significant bacterial activity and horizontal gene transfer. The source of these primary colonists remains somewhat obscure but in studies of developing seedlings the bacteria identified are often typical of those also found in the spermosphere and in rhizosphere soils. As plants develop the phyllosphere community develops and becomes distinct, both in terms of relative abundance and complexity, from that found in the below ground environment. For example, common rhizosphere and soil associated bacteria such as those of the genera *Rhizobium* or *Azospirillum* do not effectively colonise leaves^{38, 83} and with few exceptions¹¹⁰ plant growth promoting bacteria (PGPB), particularly *Pseudomonas* and *Xanthomonas* isolates, establish very limited and transient leaf population densities when compared to roots following seed or soil inoculation²⁴. Indeed even direct topical application of bacterial suspensions rarely establishes large persisting densities²⁹. These limited successes may be due to the method selected for the propagation of inocula in laboratory media although such strategies have proved very useful in the delivery of biological functional traits for transient plant protection; for example in the reduction of insect pest damage by *Bacillus thuringiensis*, the use of *Klebsiella* spp. or *Beijerinckia* spp. nitrogen fixing bacteria as biofertilisers and the limitation of frost damage by ice nucleating bacteria⁶³.

There are few examples of successful direct application of inocula to leaves that result in established active colonising populations of bacteria. One detailed study⁶¹ followed the point source transfer of an established phyllosphere bacterial population, a natural isolate *P. fluorescens* SBW25 that carried novel genetic markers. Effective bacterial transfer from leaf to leaf was observed which established persisting and colonising populations of transferred

bacteria on the emerging leaves of adjacent field grown plants. Direct leaf to leaf physical contact or rain splash were not effective and only resulted in a transient contamination. However, the introduced phytophagous lepidopteran pest *Mamestra brassicae* was an effective vector of bacteria. The lepidopteran transferred inocula increased density, established and colonised emerging leaves and developing leaves equivalent to the normal carrying capacity and phyllosphere density observed for seed inoculated SBW25^{3, 61}.

Assessment of the true extent of dispersal and distribution of natural populations is limited by the practical restraints associated with the collection of representative samples of sufficient magnitude to allow assessment, and the sensitivity of analytical procedures^{33, 34, 41, 42}. Methods that allow detailed comparisons are essentially molecular in character and provide either genotypic or phenotypic signatures. These include metabolic and molecular techniques that have been employed for the identification of isolates: BIOLOG²² restriction digestion (t-RFLP) or direct sequencing of 16S ribosomal RNA genes, polypeptide profiling by SDS-PAGE¹⁰⁶ as well as typing using Enterobacterial Repetitive Intergenic Consensus Sequence-PCR (ERIC-PCR)^{119, 120}, Random Amplified Polymorphic DNA (RAPD)¹²³, Fatty Acid Methyl Esters (FAME) analysis^{94, 109}, Multi Loci Enzyme Electrophoresis²⁷, or specific antisera and immunofluorescence microscopy¹²⁴. In addition evaluation of total phyllosphere community composition can be undertaken with established techniques based on the common use of phylogenies based on 16S ribosomal RNA gene sequence. This approach has the benefit of simplifying description and identification as it draws on established databases. These methods rely on the use of highly conserved oligonucleotide primers for general description or the use and application of group or taxon specific oligonucleotides. The advantage is that total cell extracts or total nucleic extracts can be examined to estimate diversity or relative abundance without the need to have to isolate bacterial colonies by traditional methods. Molecular techniques have revolutionised our ability to describe the diversity of communities, predominantly on the basis of 16S ribosomal RNA gene phylogeny. An advantage of molecular techniques is that they can be developed to analyse the total nucleic acid fraction (DNA and/or RNA) extracted directly from the environment. Methods include reverse array hybridisation and clone library screening, PCR-based community profiling techniques such as denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE)⁷⁹. Other methods based on PCR include amplified ribosomal DNA restriction analysis (ARDRA), terminal restriction fragment length polymorphism (T-RFLP) and length heterogeneity LH-PCR which are all based on enzymatic digestion of PCR derived amplimers with restriction endonuclease enzymes⁹⁴. In one of the first applications of its kind²⁸ applied DGGE-16S rRNA gene analysis was applied to describe the total diversity of phyllosphere

communities. Related studies¹²⁵ confirmed their findings that many previously described bacterial genera are abundant and that few novel taxa can be detected. As seen in many rhizosphere studies¹⁰⁹ phyllosphere diversity also varies between plant species and time of sampling⁴³, but leaf associated communities were less complex.

These now well established direct approaches for assessing diversity of isolates or total communities can be complemented by methods that target suspensions of cells removed from leaves, for example, Fluorescence in situ hybridisation (FISH) and immunofluorescence⁵¹. As these methods target particular bacterial types (or related groups) in suspensions of whole cells they are useful in determining the relative abundance of community components. Cells can then be enumerated by epifluorescence microscopy and image analysis to correlate abundance with cellular activity¹²¹ or activity and abundance can be evaluated by the use of fluorescence activated cell sorting^{113, 122}. Additional strategies abound for the study of inocula introduced to the phyllosphere and include the utility of genetic manipulation for the identification of in vivo induced promoters^{6, 70, 90}. The original ice work of Lindow^{64, 65}, the use of the *lacZY* system and other reporter genes have allowed the monitoring of inocula³⁶ on plant surfaces or specifically in the phyllosphere. Many approaches have been applied to monitor fluorescent pseudomonads following field release to study the ecology of inocula under “natural” conditions, as biological control or plant growth promoting inocula and in biosafety studies to assess impact, evaluate gene stability and determine the potential for horizontal gene transfer in the natural environment^{4, 5, 7, 55, 56}. More recently GFP, or its variants, have been applied to provide better methods for in situ detection and for the direct monitoring of the distribution and activity of individual cells. The use of GFP has advantages over the use of immunofluorescence, bioluminescence and other markers, in that detection can be noninvasive and highly sensitive³⁵. How the use of these molecular approaches and the application of reporter systems in genetically modified inocula have advanced our understanding of phyllosphere microbiology will be discussed in further detail below. Before these aspects are discussed it must be remembered that traditional microbiological methods based on the isolation, enumeration and identification of populations that are able to grow and form colonies under laboratory conditions has been the mainstay of phyllosphere biology. Plate count methods are of particular value, selective and specialist media are available that facilitate the isolation of specific groups, notably the pseudomonads^{16, 29, 108}. Although it is generally acknowledged that only a proportion of the total phyllosphere community (estimates range from 0.1–30% of all bacterial cells present) can be grown in the laboratory, the case has not been proven to demonstrate that these traditional approaches are any less valuable or less representative than molecular methods for characterising

community dynamics. They are just more laborious and less fashionable. Where traditional methods have been compared with other methods, for example in the tracking and monitoring of genetically modified inocula carrying reporter genes, plate counting has been shown to be more sensitive and accurate. With the exception that plate count methods cannot directly report on the in situ activity of the inocula.

5. COMMUNITY DIVERSITY AND SUCCESSION IN THE PHYLLOSHERE

The size and nature of the phyllosphere microbial community is not only a reflection of the host plant physiology and local environmental conditions, but also the phenotypic and genotypic plasticity of local microbial populations and how they adapt to change⁹². If a population cannot adapt it will be out competed by succeeding groups, this event may be dynamic and diurnal cycles as well as seasonal cycles of succession have been observed. In studies of the seasonal changes seen in the phyllosphere of sugar beet^{2, 108, 109} greatest microbial numbers were detected on senescing primary leaves. This study also showed that fluctuations in numbers of filamentous fungi, yeast and bacteria varied in a similar way on senescent primary, mature and immature leaves. Population densities increased from day 1 to a peak 114 days after planting and then gradually decreased. This study showed that the phyllosphere is a highly selective habitat for microorganisms, with as few as 13 dominant bacterial species (from replicated samples of 3×30) found on each leaf type over a growing season, and these communities fluctuate diurnally¹⁰⁸. And that the bacterial community on any given leaf type on a given day was usually numerically dominated by one or other of 5 species. A few detailed studies have followed community diversity and succession in both space and time^{29, 64, 65}. In a related detailed study of the leaves of olive trees over consecutive seasons studied for 6 years Ercolani recorded the diversity of aerobic heterotrophs²³. The main observation made was that distinct patterns of colonisation were also observed at different sampling times indicating strong selection and succession. What is interesting in these and related studies, is the estimate of variation in temporal and spatial diversity. Both abundance and community diversity are often highest in emerging or senescing leaves, due no doubt to nutrient resource availability. However, in established or mature leaves both the total count and diversity of bacterial populations is considerably lower. This low bacterial diversity has been seen in other studies, in particular considerably reduced numbers and relative diversity are observed in *Gramminaceae* and waxy-leaved plants when compared to broad leaf plants^{9, 42, 50}. Bacterial community diversity was at its lowest mid season correlating with the warmest driest weather and at its highest near the

end of the season when rainfall was high and temperature low. Other studies on sugar beet have shown bacterial proliferation and recolonisation from season to season, emphasising the point that phyllosphere colonists are specialists²¹. These observations have led to more detailed molecular investigations to establish whether bacteria colonising aerial plant surfaces carry novel genes specific to that habitat and how bacteria perceive the habitat and regulate adventitious gene expression.

In vivo expression technologies (IVET)^{70, 90} have been applied as a gene fusion technology to identify ecologically significant genes on the basis of their positive contribution towards a specific phenotype. This has been used to study environmentally significant genes expressed in the phytosphere by non-pathogenic plant associated bacteria such as *P. fluorescens* SBW25^{89, 90}. Interestingly this method identified a promoter in SBW25 associated with a type III (*hrp*) gene cluster⁸⁹ which was closely related to the operon common in the phytopathogen *P. syringae*, although the expression of *hrp* in the phyllosphere by SBW25 has not been confirmed. The detailed studies of Hirano and Upper working with *hrp* mutants of *P. syringae*^{29, 30, 31} confirmed that the complete type III secretion pathway is essential for colonisation and growth in the phyllosphere. How these interact with plant systems and cells has yet to be resolved. But it is clear that type III pathways are not unique to pathogens as the non-pathogenic bacteria *P. fluorescens* SBW25 and *P. putida* KT2440 also have almost complete *hrp* operons⁸⁹ illustrative that components of this gene cluster are a key set of ecologically significant genes necessary for phyllosphere fitness. In related studies it has been shown that plasmids representative of the horizontal gene pool accessible to SBW25 and other phyllosphere *Pseudomonas* spp. (see below) also carry ecologically significant genes that are induced to express only in the phyllosphere⁶. These studies confirm the importance of plasmids in host ecology as they carry unique traits that contribute to host fitness and local adaptation to the niche.

6. THE ROLE OF THE HORIZONTAL GENE POOL IN ADAPTATION TO THE NICHE: A CASE STUDY

There have been a number of reports of the specific role of extra-chromosomal self replication genetic elements, or plasmids, in local adaptation. In particular, resistance to a number of bacteriocidal agents, including antibiotics such as streptomycin, mercury and copper, were observed in Gram-negative bacterial pathogens in a variety of crops^{4, 10, 15}. Resistance strains

were selected for by treatment and in many instances resistance was transferable from one bacterial isolate to another. The abundance and diversity of plasmids in phyllosphere microbial communities is well established^{3, 4, 6, 47, 88}. The phyllosphere also provides a suitable environment for the conjugative transfer of plasmids between either natural populations or introduced populations of colonising bacteria. Indeed, under certain conditions, the frequency with which plasmid transfer takes place can be induced at the leaf surface. Transformation, or the active uptake of cell-free DNA by competent recipient bacteria, is also promoted in decaying or diseased plant tissue^{39, 80, 81}. In the latter studies plants were infected with *Ralstonia solanacearum*, causative agent of bacterial wilt and *Acinetobacter* sp.BD41. These bacteria were introduced as donor and recipient of a marker gene cassette carried by transgenic tobacco plants. The recipient bacterium BD41, carried a deletion derivative of the selectable antibiotic resistance marker, *aadA*, on either its chromosome or a multicopy plasmid. Therefore following the transformation of the recipient and uptake of DNA from the recipient a homologous recombination event occurred and a functional copy of the marker gene was transferred. The relevant finding was that transformation frequencies observed in the vascular tissue were many orders of magnitude greater than expected. Unexpectedly the recipient bacterium established significantly greater densities when inoculated with *R. solanacearum* than it did when inoculated alone. Also the recipient strain developed a greater level of competence in planta than was typically achieved in vitro. This ability for enhanced frequencies of gene transfer is typical of the phytosphere. As mentioned above conjugation is also stimulated in the leaf habitat. Studies with natural isolates of bacteria and their indigenous plasmids collected from sugar beet plants transferred at 100× greater frequencies in planta than in vitro^{56, 58, 60}. In addition there is good evidence for the abundance of bacteriophage in the phyllosphere, both as a source of genetic variation and as a key component in regulating population densities, in fact predator – prey interaction between phage and their host populations may be a key factor in driving succession and promoting population crashes².

Horizontal transfer of genes has an important role in the adaptation of many bacterial populations^{8, 52, 62, 107}, and novel genes that enter the horizontal gene pool may have unexpected effects that are difficult to predict. As field experiments with GMMs are often constrained, few ecological investigations have studied horizontal gene transfer under natural conditions^{19, 55, 56, 110, 118}. A practical alternative is to work in microcosms that replicate many of the features of natural habitats^{20, 58, 95}. Therefore a detailed study of population dynamics and gene flow in pseudomonad phytosphere communities was undertaken using environmental growth chambers that mimic the biota and climatic conditions typical of the margin of an agricultural field⁵⁸. The studies were undertaken at the UK Natural Environment Research Council's Ecotron

Facility housed at Silwood Park, Ascot. This facility consists of 16 controlled environment chambers, in two arrays of eight, in which the light, temperature and moisture regime are controlled to mimic ambient field conditions⁴⁹. In each chamber model plant communities are grown in soil with their associated above and below ground fauna. The plant communities consisted of four weed species (*Senecio vulgaris*, *Conyza canadensis*, *Stellaria media*, *Veronica persica*) introduced into a sandy loam soil supplemented with a leaf-litter mulch on the surface. Physical conditions of light, temperature and humidity approximated that of a typical English summer's day, "cloudy with regular rain showers!"

6.1. Population Dynamics of Inocula in Chickweed

This study was undertaken as laboratory and field based studies have shown the phytosphere to be a hotspot for horizontal gene transfer mediated by plasmids^{14, 18, 46, 48, 60, 82, 97, 101, 114, 115, 117} by bacteriophage^{2, 40} and by natural transformation^{12, 39, 80, 81}. Therefore we established communities in the phyllosphere of *Stellaria media* (chickweed) followed by seed inoculation with 5×10^4 cfu of different variants of the well-studied phyllosphere bacterium *P. fluorescens* SBW25 which was originally isolated from sugar beet leaves^{5, 91}. In previous field release studies conducted over two seasons we had already demonstrated that a marked variant of this bacterium colonised the leaves and roots of seed inoculated sugar beet to establish normal population densities up to the carrying capacity for pseudomonads^{53, 108}. As anticipated no transfer of the chromosomally located marker cassette to indigenous pseudomonads was observed. Furthermore, the genome architecture of the bacterium remained stable indicating that reassortment, loss or rearrangement of the chromosome was not a typical event. This limited study implies chromosome stability, and that the extensive genetic diversity recorded for pseudomonad populations results from expansion and contraction of "chromosomally genetically stable" ecotypes. However one important discovery made was that introduced strains naturally acquired conjugative plasmids from the indigenous phyllosphere community at specific phases of sugar beet development⁵⁵. Interestingly these horizontal gene transfer events coincided with a period when carriage of these indigenous plasmids had a selective advantage⁵⁶. These data and those from related studies of plasmid presence in other natural leaf pseudomonads, and that plasmid transfer frequencies between donors and recipients introduced to the leaf surface are enhanced, are taken to illustrate that the horizontal gene pool is an essential component for adaptation in these communities. Our own work demonstrates that components of the standing HGP as represented by plasmids carry unique accessory genes that are responsive to plant and bacterial cues for de novo expression. One well-studied plasmid, pQBR103 carries

many novel open reading frames not present on current genome databases and quite distinct from the many different *Pseudomonad* genes identified as a result of recent genome sequencing activities (see ref. 103 for details of the SBW25 and pQBR plasmid genome web sites). The ability to gain and lose quasi-essential genes that facilitate niche exploitation is not a novel concept; however, the reason that these habitat specific genes are retained on plasmids, rather than being incorporated into the chromosome remains unresolved. These issues form a part of wider ecological and evolutionary questions that have been considered elsewhere (112 and references there in).

Detail has been provided of the transfer studies undertaken in the highly controlled environment facilities undertaken in the Ecotron⁵⁸. This investigation compared the population dynamics of *P. fluorescens* SBW25 carrying a presumed neutral marker gene cassette (KX (*aph*, *xylE*)⁵, inserted: (a) in the bacterial chromosome, (b) in the chromosome in the presence of a transducing lysogenic-phage isolated from the sugar beet phyllosphere (Φ 101); and (c) in a natural transfer proficient (*tra*⁺) plasmid pQBR11 (pQBR11 is closely related to pQBR103—see above). The frequency of gene transfer was monitored from inocula, as was the frequency of the transfer of mercury resistance plasmids from the indigenous community to the naturalised inocula. The spatial and temporal population dynamics of the three inocula, the unmodified control SBW25R (spontaneous rifampicin resistant mutant), SBW25R::KX (*rif*^r, *kan*^r, C2, 3O⁺), SBW25R::KX-(Φ 101) (*rif*^r, *kan*^r, C2, 3O⁺) and SBW25R(pQBR11::KX) (*rif*^r; *tra*⁺, HgCl₂^r, *kan*^r, C2, 3O⁺) were compared by standard selective plate isolation methods using appropriate antibiotics of leaf samples collected over the growing cycle of the colonised chickweed.

To assess the transfer of the KX gene cassette to the indigenous bacteria, plant homogenates were plated on PSA-CFC agar supplemented with kanamycin (75 μ g ml⁻¹) and resultant colonies assayed for catechol 2,3 dioxygenase activity. Sensitivity of detection was increased using SBW25 specific phage counterselection⁵⁸. The frequency of transfer of native mercury resistance plasmids from the indigenous community to the SBW25R inocula was determined in SBW25R and SBW25::KX treatments only. Although mercuric resistance is commonly associated with mobile genetic elements^{59, 84} it is not a ubiquitous trait in phyllosphere bacteria^{47, 88}. An appropriate sub-sample of isolated colonies were compared by the REP-PCR genotyping method^{119, 120} and selected indigenous recipients of pQBR11::KX were identified by sequence analysis of 16S ribosomal RNA genes using standard methods (Figure 1).

The population densities of all inocula increased between days 5 and 11 on seedlings ($F_{1,8} = 5.56$, $p = 0.046$). No significant difference was observed between treatments, and no interaction between treatment and time was recorded. Inocula densities on *S. media* continued to increase between

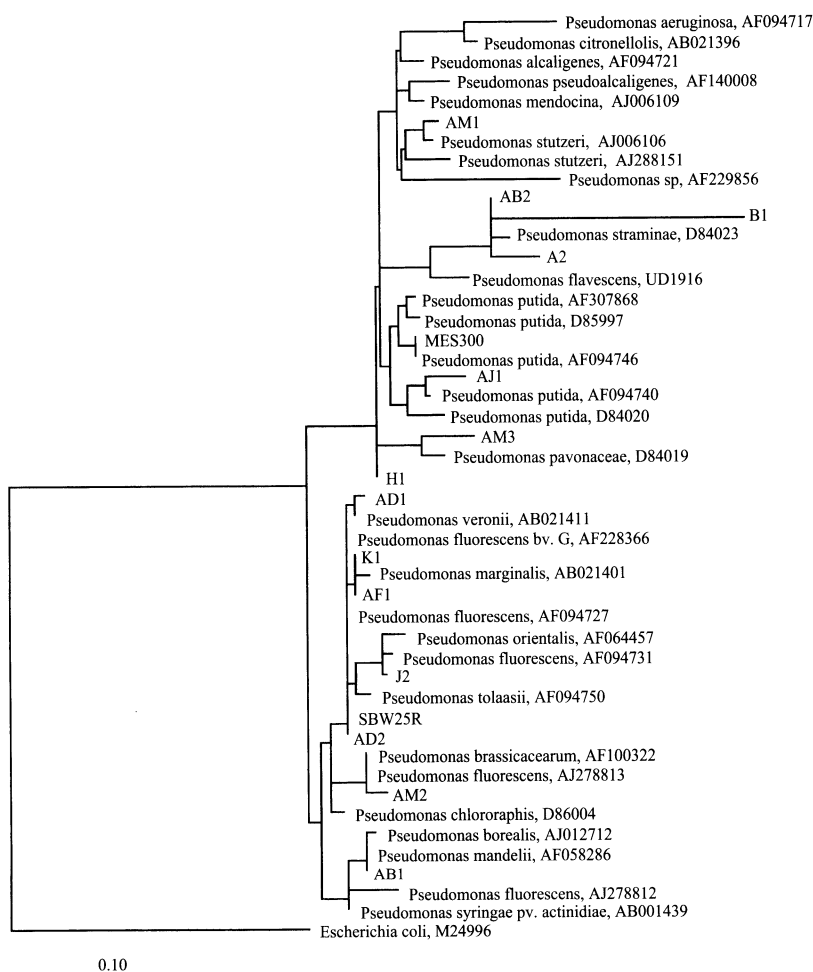


Figure 1. Illustration of the extent of diversity of phyllosphere communities based on 16S rRNA sequence analysis. The dendrogram shows the phylogentic distribution of 14 natural isolates isolated from chickweed leaves that had acquired pQBR11 in situ. Transconjugants were first observed 75 days after the donor *P. fluorescens* SBW25 (pQBR11::KX) had been introduced as a seed dressing and established a “naturalised community” on chickweed. The apparent delay in active HGT is a typical observation for these types of plasmids⁵⁶. Data amended from⁵⁸.

days 18 and 25 except for the phage treatment, which fell slightly. Samples taken from the bacterial communities established on leaves on four occasions between days 53 and 95 showed a strong effect of treatment on bacterial densities on the leaves ($F_{3,10} = 192.27$, $p < 10^{-4}$) with post hoc comparisons showing sharp decline in those communities carrying the lyso-genic phage which fell below detectable limits (50 cfu g⁻¹) by day 88.

Densities of the pQBR11::KX carrying inocula increased with time in the phyllosphere ($FPB_{3,8} = 17.73$, $p = 0.0007$) to match the SBW5R control, which stabilised at $c. >10^5$ cfu g⁻¹ by day 75. These patterns of apparently low initial fitness followed by selection for plasmid presence were similar to those we have observed in field studies with sugar beet⁵⁵. In the field investigations with bacteria indigenous to sugar beet plants and the study field in Oxfordshire, United Kingdom we observed a periodic increase in the population density of plasmid carrying strains of some five orders of magnitude coincident with plant maturation. The selective factors expressed by the plasmid that led to proliferation are still under investigation, but we have identified UV resistance determinants and functions relevant to RNA turnover and cell survival. These studies have allowed us to determine a turnover or doubling time for the fluorescent pseudomonad of approximately 30 hr. This correlates for turnover periodicity for components of the phyllosphere community of 48 hr as assessed by Ellis *et al.*²¹ who studied genotype turnover and persistence of this group. We also estimated rates of gene flow of the introduced marked plasmid (pQBR11::KX) to indigenous pseudomonads. Again transfer coincided with selective proliferation of inocula carrying the plasmid 74 days after sowing. Transfer was recorded in three out of the four replicate chambers in this treatment. The highest transconjugant density (indigenous recipient bacteria with acquired marked plasmid) was 360 cfu g⁻¹, equivalent to 4.2% of the actual donor population established in the phytosphere. This value is considerably higher than would be expected from the equivalent figures of $1 \times 10^{-2}\%$ to $1 \times 10^{-4}\%$ observed in the in vitro plasmid transfer assays (Box 1) used for testing transfer frequencies prior to seed inoculation (Table 1).

Plasmid transfer and fitness model.

Transfer rate and fitness parameters were estimated for transfer of plasmids to and from the inocula. Two models were established, fitted to the data and simulations run to investigate realistic values for plasmid transfer and their interaction with the affects of plasmid acquisition on host fitness. The models were:

Model 1.

$$\frac{dD}{dt} = \mu_D \cdot D \cdot C_D \quad \frac{dTC}{dt} = \mu_{TC} \cdot TC \cdot C_{TC} + k_D \cdot (D + TC) \cdot C_{TC}$$

Model 2.

$$\frac{dR}{dt} = \mu_R \cdot R \cdot C_R \quad \frac{dTC}{dt} = \mu_{TC} \cdot TC \cdot C_{TC} + k_{DR} \cdot (D + TC) \cdot R \cdot C_{TC}$$

Where D, R and TC are the donor, recipient and transconjugant counts (cfu g⁻¹). Their rates of change (growth) are indicated by μ with the appropriate subscript. The letter C_{subscript} is the logistic parameter fitted to constrain each of these populations to the final sizes observed. This is similar to defining a carrying capacity. Using the donor population as an example this took the form: $C_D = (1 - \text{donor population/final donor count})$. In one instance the simple logistic growth function would not provide an acceptable fit and the model was modified by inclusion of power functions. In both models μ_{TC} is the sum of two components, the first (μ_R) was the estimated rate of change of the recipient population and the second (α) was the additional effect of plasmid carriage on the rate of change in density of the transconjugant population.

Both models assume that all donors and transconjugants can transfer plasmids at similar rates and that new transconjugants do not have significant lag times before they become competent donors.

Table 1. Plasmid transfer coefficients and equivalent plasmid fitness effects (α).

Transfer coefficients ^a	Leaf	Transfer coefficients ^b	Leaf
K_D (TC D ⁻¹ h ⁻¹)	α h ⁻¹	K_D (TC D ⁻¹ h ⁻¹)	α h ⁻¹
1×10^{-4}	0	1×10^{-4}	0
1×10^{-5}	0.005	1×10^{-5}	0
1×10^{-6}	0.015	1×10^{-6}	0.01
1×10^{-7}	0.020	1×10^{-7}	0.015
1×10^{-8}	0.025	1×10^{-8}	0.02
K_{DR} (g cell ⁻¹ h ⁻¹)	α h ⁻¹	K_{DR} (g cell ⁻¹ h ⁻¹)	α h ⁻¹
1×10^{-9}	-0.3	1×10^{-9}	0
1×10^{-10}	0	1×10^{-10}	0.01
1×10^{-12}	0.02	1×10^{-12}	0.02
1×10^{-14}	0.03	1×10^{-14}	0.03
1×10^{-16}	0.04	1×10^{-16}	0.04
1×10^{-18}	0.05	1×10^{-18}	0.05

^aTransfer of plasmid pQBR11::KX from *P. fluorescens* SBW25R to indigenous pseudomonads on *S. media* leaves. Here, α is the additional growth rate that plasmid carriage must confer on recipients if these transfer rates are to be fitted to the observed data.

^bAcquisition by SBW25R of mercuric-resistant plasmids from indigenous pseudomonad community on *S. media* leaves. Here, α is the additional growth rate that plasmid carriage must confer on SBW25R recipients if these transfer rates are to be fitted to the observed data.

In model 1, k_D is a simple rate parameter in which the rate of plasmid transfer is taken as a simple function of the number of donors present. In model 2, k_{DR} is a second order rate constant in which the rate of plasmid transfer is a function of the multiple of the numbers of donors and recipients present, that is, a function of (D • R). This is analogous to the plasmid transfer

coefficient k_{tl} ⁵⁴. Strictly speaking this is a mass action parameter, requiring thorough mixing of donors and recipients. However, this has been successfully applied to measurement in biofilms^{67, 98, 100} and to studies on leaves and roots^{46, 82, 96, 104}. Given values of k_D or k_D or k_{DR} were inserted into the models and values of α which gave the best fit of the model to the observed data were fitted iteratively until the sums of squares of the difference between the model value and the geometric means of the observed data were minimised. Model simulations were run using ModelMaker Ver 3.0.3 software (Cherwell Scientific, Oxford, United Kingdom). ModelMaker solved the differential equations numerically using the Runge-Kutta method.

The two models were applied to test a range of transfer rates (k_D and k_{DR}) and determine accordingly the change in host fitness (α) required to achieve the observed transfer of pQBR11::KX. For modelling, the recipient population was estimated as one tenth of the observed total pseudomonad count ($n = 14$ – 16 replicates per sampling). The donor population (SBW25R pQBR11::KX) was evaluated from selective isolation plates ($n = 4$ replicates per sampling). The donor and recipient logistic growth curves were fitted first to provide values of μ and C for the models. The model simulations were started at day 53 when no transfer was detected and ran to day 95. The transfer rate parameters were tested in tenfold steps between 10^{-4} and 10^{-8} (k_D) and between 10^{-9} and 10^{-18} (k_{DR}). For each transfer rate value, a value of α (additional growth rate conferred by plasmid carriage) was fitted iteratively to achieve the best fit of the model to the data. At the lowest transfer rates considered ($k_D = 10^{-8}$ TC D⁻¹ h⁻¹, $k_{DR} = 10^{-18}$ g cell⁻¹ h⁻¹) the maximum required fitness benefits of plasmid carriage (α) on root and leaf are 0.05 h⁻¹, equivalent to an additional cell division, new generation, every 20 hr. At the highest transfer rates considered ($k_D = 10^{-4}$ TC D⁻¹ h⁻¹, $k_{DR} = 10^{-9}$ g cell⁻¹ h⁻¹) the number of transconjugants observed on leaves could still have been generated even if the plasmid had no effect or a negative effect on a substantial burden ($\alpha = -0.3$ h⁻¹). These predictions match the values observed in the field release experiments with SBW25 and pQBR103 discussed above^{3, 55, 56}.

The increase in transconjugant counts observed in the chickweed investigation is considered to be a function of plasmid transfer and growth. The transconjugant population values did not increase indefinitely but achieved a density interpreted as the carrying capacities for those populations in the prevailing conditions. As well as monitoring the horizontal transfer of the introduced mobile elements, the reciprocal movement of mercury resistance plasmids from the natural community to inoculated bacteria was recorded on days 74, 88 and 95 respectively. The percentage of the population of inocula that acquired indigenous plasmids varied from 0 to 35%. For modelling purposes, the donor population was taken as the mercuric-resistant pseudomonad counts. From our collection, a random sample of

116 mercuric-resistant pseudomonads isolated from *S. media* in the Ecotron were assayed using standard methods⁵⁹ and 86 (74%) of these transferred mercuric resistance to SBW25R. For each transfer rate value, a value for α (additional growth rate conferred by plasmid carriage) was fitted iteratively to achieve the best fit of the model to the data. Transfer rate values and their equivalent changes in host fitness are given in Table 1. At the lowest transfer rates considered ($k_D = 10^{-9}$ TC D⁻¹ h⁻¹, $k_{DR} = 10^{-18}$ g cell⁻¹ h⁻¹) the maximum required fitness benefits of plasmid carriage (α) on root and leaf are 0.04 h⁻¹ which is equivalent to a doubling time of 25 hr (Table 1B).

REP-PCR fingerprint analysis of isolates was applied to provide a snapshot of the extent of the natural diversity of the pseudomonad populations. From four sampling dates, 326 randomly sampled pseudomonad isolates were purified and assigned to 176 fingerprint types (FPTs). These 326 isolates, included 116 which were naturally resistant to HgCl₂ (mer^r) and were represented by 69 FPTs. Of 176 FPTs, 36 were isolated twice and the remainder isolated only once accounting for 47% of all isolates. The six most common FPTs were represented by nine to twelve isolates, 19% of all isolates. No FPTs were isolated on more than one sampling date, however, the same FPT were isolated from more than one Ecotron chamber demonstrating their wide distribution and specialised to the phyllosphere. The 16S ribosomal RNA gene sequence analysis of a random collection of 14 of the indigenous phylloplane isolates that had acquired the introduced plasmid (pQBR11::KX) illustrates not only the diversity of ecotypes present, but also the extent of the plasmid host range in *Pseudomonas* spp. (Figure 1).

The transfer frequencies of the marked plasmid, pQBR11::KX to indigenous pseudomonads are comparable with those seen in other studies of plasmid transfer to bacterial communities. These have typically focussed on the broad host range, RP4 or similar incP-type antibiotic-resistance plasmids which have high transfer frequencies^{18, 25, 91, 99}. In the wheat rhizosphere, Smit *et al.*^{101, 102} observed the transfer of plasmid RP4 from *P. fluorescens* to indigenous bacteria at frequencies of up to 5×10^{-4} TC/D (transconjugants donor⁻¹). Richaume *et al.*⁹³ recorded transfer frequencies of up to 10^{-1} TC/D for plasmid RP1 transfer from *P. syringae* to indigenous populations on bean foliage maintained at high humidity and plasmid R68.45 transfer from *P. aeruginosa* to indigenous soil bacteria ranged from 10^{-6} to 10^{-2} TC/D²⁶. These studies were not undertaken with 'naturalised' populations. The in planta studies undertaken with pQBR11::KX revealed transfer frequencies (TC/D) between naturally established populations of up to 100 times greater than those observed in vitro. These data support our previous short-term investigations of the influence of the surface of sugar beet roots on stimulating transfer⁶⁰ and the findings of Normander *et al.*⁸², and Björklöf *et al.*^{13, 14} who observed in planta transfer of the TOL and RP1 plasmids to be up to 30 times greater than

in vitro estimations. The higher transfer rates may be promoted by the aggregation of pseudomonads in favourable microhabitats, and the nutritional flow of plant exudates. A difficulty with this type of study is distinguishing plasmid spread via conjugation (horizontal spread) and through the proliferation of transconjugants (vertical spread). We have addressed this through models which investigate the relationship between the horizontal (k_D , k_{DR}) and vertical (α) components. Our approach has been to say, if the plasmids were transferring at a specific rate, then, what effect would the plasmids have to have had on host fitness to produce the observed data?

The data indicate that in most conditions plasmid pQBR11::KX cannot proliferate to the extent observed without conferring some growth benefit to the host. At the relatively high transfer rate of $k_D = 1 \times 10^{-4} \text{ TC D}^{-1} \text{ h}^{-1}$, transfer alone could sustain the transconjugant population. Higher values of k_D could also sustain this population even when fitness costs are incurred from plasmid carriage. It is noticeable that even at low transfer rates $k_D = 1 \times 10^{-8} \text{ TC D}^{-1} \text{ h}^{-1}$, the fitness benefits necessary to sustain the observed transfer would have been modest with α less than 0.03 h^{-1} . The k_{DR} values may be compared with k_{II} values in other rhizosphere and phyllosphere studies where values of 4×10^{-11} to 2×10^{-13} (ref. [94]), of 10^{-14} to 10^{-9} (ref. [104]) and up to 6×10^{-14} Knudsen *et al.*⁴⁶ have been reported. The conditions employed, probably ensured that these values are close to their upper limits. Normander *et al.*⁸² for example report exceptionally high k_{II} values up to 1.4×10^{-7} for transfer of a TOL plasmid derivative on bean leaves at 100% relative humidity. These studies have fitted models in which the effects of plasmid carriage on host fitness are fixed and usually considered to be neutral. Generally, however, plasmids impose costs (associated with carriage and transfer) on their hosts, but can also confer periodic benefits associated with plasmid encoded traits⁵³. Our study is first to investigate the relationship between transfer rates (horizontal component) and the affects of plasmid carriage on host fitness (vertical component) using realistic phytosphere data.

With second order model of transfer and k_{DR} values in the order of 10^{-9} it is apparent that pQBR11::KX could conceivably sustain the transfer rates observed to maintain transconjugant densities if the effect of plasmid carriage was neutral on host fitness. This value is, however, high and it is unlikely that the conditions are present to reach this value in natural habitats such as the phytosphere⁹⁹. Our in vitro biofilm studies have observed pQBR11 k_{II} values ranging 10^{-12} to 10^{-15} in various conditions⁵⁷ indicating that a typical value for this plasmid in a phytosphere environment may be $\sim 10^{-14}$. At such a value our results indicate that the plasmid would have to compensate for the costs of carriage and increase the host growth by 0.03 h^{-1} , that is, the equivalent of an additional replication compared to a plasmid free bacterium every 33 hr.

This value correlates with the direct estimates of doubling time for the plant directed selection of plasmid carrying inocula made in sugar beet^{3, 55, 56}.

In the chickweed phytosphere the recipient population density was observed to be either stationary, or rising or falling slowly. This population of pseudomonads will have included many sub populations which may individually have been rising or falling more sharply. Plasmid pQBR11::KX could potentially have benefited from transfer to sub-populations which were growing more actively¹¹. This possibility was considered in our investigation using REP-PCR fingerprinting to identify whether plasmid transfer was associated with the changing predominance of specific populations. This method identified a high diversity of pseudomonad FPTs and correlated with the observations of others¹⁶. This diversity was similarly high among transconjugants and no specific types were predominantly associated with the plasmid flux.

No transfer of the gene cassette from the chromosome was observed despite the high population densities achieved by these bacteria, and the acquisition of indigenous plasmids and phage. This was anticipated given the zero or low frequencies of *Pseudomonas* spp. chromosomal gene transfer observed, under optimal conditions in the laboratory⁵, in wheat rhizosphere microcosms¹¹¹ or in barley seedling microcosms⁹⁷. Even using plasmids that were able to mediate chromosome transfer to complement auxotrophic strains¹¹¹, frequencies of the order of only 1×10^{-6} prototrophic chromosomal recombinants per donor were observed. These rates are likely to be lower under natural field conditions where bacterial population densities, growth rates and metabolic activity are all reduced.

7. CONCLUDING REMARKS

The phyllosphere is a special environment that deserves greater attention. It is accessible, ubiquitous and the keeper of many interesting microbial functions. We have much to learn about bacterial evolution and ecology. The leaf surface can provide a habitat for the direct study of how the plasticity of the genome, the diversity of mixed communities and the complexity of aggregates maintains and defines the diversity of functional microbial communities. Why is the diversity so extensive, why are plasmids and bacteriophage so abundant? How do bacteria regulate and perceive their neighbours and environment? What defines a pathogen and an epiphyte? Indeed, are phyllosphere bacteria as important to plant growth promotion as rhizosphere bacteria appear to be? How specific and specialised are the communities—phylloplane specialists or plant species specialist? All in all the phyllosphere appears to have the variety and challenging questions necessary to attract and fully occupy microbial ecologists, geneticist and population biologist for many years to come.

Pseudomonads have provided a very tractable study group—yet there is still much to learn about any one single isolate.

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LIFE AS A BIOCONTROL PSEUDOMONAD

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1. INTRODUCTION

Many *Pseudomonas* strains can benefit plants directly, by promoting plant growth and health, and/or indirectly through inhibition of, or competition with pathogens, parasites, or plant competitors^{21, 32, 39, 43, 60, 79}. Not all pseudomonads have biocontrol capacities, and among those that do certain display several different biocontrol mechanisms. Current *Pseudomonas* taxonomy is not satisfactory and requires extensive revision^{9, 86}, but from the existing knowledge it appears that biocontrol capacities are rather strain-dependent than species-dependent. The significance of biocontrol pseudomonads for plant protection is exemplified by their contribution to disease-suppressiveness of soils^{62, 65, 112}. This chapter provides an overview of the ecological particularities of biocontrol pseudomonads, as well as a review of interaction mechanisms with phytopathogens, the plant, and non-target soil microbiota.

2. THE PEREGRINATION OF BIOCONTROL PSEUDOMONADS

2.1. Life Through a Succession of Different Microbial Habitats

Biotic interactions important for suppression of phytoparasites take place when biocontrol pseudomonads are plant-associated, and to a lesser extent in soil prior to their colonization of the plant²⁰. Consequently, research has mainly focused on the ecology of biocontrol pseudomonads during the interaction of the latter with the plant. Yet, an important aspect of their ecology is the succession of microbial habitats that (and thus of ecological conditions) that biocontrol pseudomonads may go through in time.

First, it is likely that biocontrol pseudomonads spend considerable time in non-rhizosphere soil, in between the death of a colonized plant and the colonization of a subsequent plant¹⁰¹. Ecological opportunities for biocontrol pseudomonads outside the rhizosphere can vary greatly. While bulk soil is usually considered an oligotrophic environment, association with particulate organic matter (e.g., plant residues) or passage through the digestive tract of soil animals may provide favourable ecological conditions for biocontrol pseudomonads to proliferate⁹¹. Their relevance to understand the distribution of biocontrol pseudomonads in non-rhizosphere soil and their consequences in terms of colonization of the following plant are not well documented. Apart from proliferation, the distribution of biocontrol pseudomonads in bulk soil will also depend on spatial dissemination. While in soil, biocontrol pseudomonads have probably limited opportunity for dissemination in most cases¹⁰⁵, except when it is assisted by members of the soil fauna^{46, 93} and/or heavy rainfall¹⁰³. In the latter case, significant vertical dissemination may indeed take place when (a) biocontrol pseudomonads are introduced to soil by inoculation (i.e., at high cell numbers), (b) soil macroporosity is well developed (thereby facilitating preferential water flow by gravity), and (c) heavy rainfall happens shortly after inoculation (i.e., before introduced cells become adsorbed to soil particles and/or are incorporated within soil aggregates)⁷⁴. The observation that biocontrol pseudomonads may reach groundwater level in certain cases raises the possibility of further spatial dissemination if the water reaches agricultural drains or is subsequently used for irrigation purposes, but data to assess this possibility is scarce.

Second, the plant itself represents a microbial habitat that is heterogeneous, both in space and time. Whereas certain biocontrol pseudomonads will colonize the seed and the root system, others may also be recovered from shoots⁸⁴. Both roots and shoots are spatially heterogeneous, with biocontrol pseudomonads present as single cells and/or microcolonies at the plant surface

depending on microhabitat conditions in the phytosphere^{8, 55}. In addition, certain biocontrol pseudomonads established in the rhizosphere and/or at the root surface may also enter the root itself and behave as endophytes¹⁰⁰. The location of these endophytes may be restricted to the intercellular space of the root cortex, but some can be found inside plant cells or colonize conductive tissues¹⁰⁰. Endophytism is probably important in biocontrol terms, as it raises the possibility that bioactive metabolites relevant for plant protection be released directly inside the root. Indeed, many biocontrol pseudomonads with the ability to confer induced systemic resistance are endophytic¹⁰⁰. In strain CHA0, the endophytic behaviour was observed less often when a mutant unable to produce the biocontrol metabolite 2,4-diacetylphloroglucinol (Phl) was used⁵.

Once associated with the plant, biocontrol pseudomonads may also experience a change of ecological conditions due to plant development and aging. As differentiation of plant tissues proceeds, the physiology of plant cells changes. For instance, in the case of biocontrol pseudomonads present at the root surface, the implication can be a modification in the amount, release site, and chemical composition of root exudates available for microbial growth and activity⁴⁰.

2.2. Succession of Ecological Opportunities

As biocontrol pseudomonads experience a succession of different microbial habitats in time, they are thus confronted to changing environmental conditions in terms of nutrient availability and more generally of ecological opportunities. This situation, both in soil and in planta, has important implications for population dynamics and physiological activity of biocontrol pseudomonads.

Unlike other taxa of plant-beneficial bacteria (e.g., *Azospirillum*; our unpublished results), fluorescent pseudomonads may be recovered from bulk soil at significant population levels throughout the year at a same site¹⁰¹. Whether this observation could also be made for biocontrol pseudomonads is not known. When considering a particular biocontrol pseudomonad, bulk soil may represent adverse conditions, sometimes leading to persistence of the strain in soil as a mixed population of culturable and non-culturable cells, including cells in a viable but non-culturable state¹⁰¹. Stress factors resulting in non-culturable cells are related to soil composition (such as low soil pH; our unpublished results), soil management (e.g., at plough pan level in farm soil⁵⁸, or climatic events¹⁰¹). For some of them, such as oxygen limitation and low redox potential, the effect of stress on biocontrol pseudomonads can be mimicked under *in vitro* conditions⁵⁶. Whether viable but non-culturable state(s) correspond(s) to an adaptive strategy or a transition to cell death in bacteria

remains to be established. However, when considering more specifically the case of biocontrol pseudomonads, a key question is whether viable but non-culturable cells can recover their former physiological activity and culturability, as shown with other taxa⁷⁸, and participate in root colonization alongside cells that have retained their culturability. Non-culturable cells of the biocontrol pseudomonad CHA0 obtained by exposure to stress did not produce detectable amounts of antifungal metabolites *in vitro* (our unpublished results).

Pseudomonads are considered efficient root colonizers. This trait has been documented with seedlings, as well as plants at the last stages of development¹⁰², when root decay results in increased release of organic compounds. This opportunistic behaviour has also been found when monitoring total fluorescent pseudomonads, and so it does not appear to be a property restricted to biocontrol pseudomonads. The ability to colonize old roots, which may promote overwintering, is an asset for colonization of a subsequent plant. Indeed, biocontrol pseudomonads can readily recolonize plant roots¹³. Once on roots, biocontrol pseudomonads tend to form microcolonies when colonizing the rhizoplane. The network of microcolonies at the root surface, in which biocontrol pseudomonads coexist with other microorganisms, can be considered a biofilm of moderate energy supply according to the scale of Wimpenny and Colasanti¹¹³. The organization of microorganisms in a biofilm has implications for biocontrol, as gene expression can differ when bacteria are present within a biofilm. Quorum sensing may be of particular significance, since (a) bacteria can reach high population levels in rhizoplane microhabitats, (b) extracellular diffusion of signal molecules is limited by plant and microbial exopolysaccharides forming the biofilm matrix (i.e., the root mucigel), thereby resulting in higher signal concentrations, and (c) certain plant metabolites can activate LuxR-type quorum sensing regulators⁹⁵. Indeed, several biocontrol pseudomonads produce and/or perceive quorum sensing-relevant signals⁵². In parallel, production of antifungal metabolites is cell density-dependent²⁸ and is regulated in certain strains by quorum sensing¹¹⁴.

2.3. Genetic Make-up for Different Microbial Habitats

Since biocontrol pseudomonads are adapted to life both in soil and in planta, the question arises as to whether this trait entails a particular genetic make-up. This question can be considered in terms of (a) the type(s) of adaptive genes necessary, (b) their mode of organization (and regulation) and (c) their evolutive history (i.e., the way they arose).

Several studies have determined whether genes important for biocontrol also contribute to plant colonization^{13, 63}, but little has been done to assess

their possible role *ex planta*. Like in the rhizosphere, the loss of phenazine production ability following Tn5 mutagenesis reduced survival of biocontrol pseudomonads in non-planted soil⁶³. Regulatory genes of secondary metabolism, such as *gacA* (global regulation of secondary metabolism) and *algU* (synthesis of sigma factor σ^E) were important for persistence and cell culturability in non-planted soil^{57, 73}, but *anr* (anaerobic regulation of arginine deiminase and nitrate reductase pathways) did not play any role in survival, even under plough pan conditions, where microaerophilic conditions prevail⁵⁸.

The dual life of biocontrol pseudomonads is reminiscent of that followed by bacteria involved in pathogenic or symbiotic interactions with the plant. For certain of these bacteria, the genes needed for the interaction are plasmid-borne, resulting in virulence or symbiotic plasmids. This situation is exemplified by the pSym of rhizobial nitrogen-fixers⁹⁷. Plasmids occur in pseudomonads¹², but are infrequent in isolates from soil or roots^{12, 51}, including in biocontrol pseudomonads. Another genetic strategy is the use of loci where mutation leads to pleiotropic effects (phase variation), as known in many parasitic bacteria⁴¹. In biocontrol pseudomonads, the *gac* mutation represents a phase variation phenomenon⁹⁰.

Genomic homeostasis in bacteria is regulated by the SOS and mismatch repair systems⁷, which respond indirectly to environmental stress. In the rhizosphere, exudation brings organic substrates, but also toxic compounds which can induce transcription of stress-response promoters⁶⁶ and perhaps this can favour mutations and/or genetic rearrangements in biocontrol pseudomonads. Indeed, genetic plasticity is a plus for efficient root colonization by biocontrol pseudomonads²². These findings suggest that microevolution could be faster in plant-associated bacteria (regardless of whether they have biocontrol properties) compared with other soil bacteria. Horizontal gene transfer plays a key role in bacteria, and has been documented for pseudomonads in soil and the rhizosphere^{25, 99}. In the case of biocontrol pseudomonads it may also concern genes implicated in biocontrol. This possibility is suggested by the observation of similar genes (e.g., *prnD*) in distantly-related taxa²⁶. Certain biocontrol genes (e.g., *phlD*) might have been acquired from the plant itself¹⁷, but if so the transfer was probably ancient, that is, before the divergence between monocots and eudicots⁸⁸.

3. THE SOCIAL LIFE OF BIOCONTROL PSEUDOMONADS

3.1. Direct Biocontrol Interactions with Phytoparasites

Direct biocontrol interactions with phytoparasites may involve competition for nutrients and/or infection sites at the root surface. In the absence of a

suitable host, several fungal pathogens persist in soil in a latent stage. In the presence of plant exudates, they germinate and infect the host at particular sites of the plant surface³⁸. Competition between a *P. putida* strain and *Pythium* spp. was evidenced for pea seed exudate compounds that trigger spore germination, and it resulted in protection of peas against *Pythium* spp.⁸⁰. Indeed, catabolic profiles of effective biocontrol pseudomonads were largely similar to that of the pathogenic fungus *P. ultimum*³², suggesting that these bacteria could compete with the pathogen for carbon substrates on the root. In addition to carbon substrates, competition between biocontrol pseudomonads and pathogenic fungi can take place for soluble ferric iron⁵³. Its significance for biocontrol is likely to be highest in alkaline soils, where iron solubility is particularly low. These examples illustrate how biocontrol can result from competition phenomena involved in the acquisition of resources necessary for plant colonization, in which case the biocontrol status of pseudomonads is directly linked with their adaptation to the plant environment.

Direct biocontrol interactions with phytoparasites may also involve antagonism. Most biocontrol pseudomonads produce one or several secondary metabolites with antimicrobial properties⁸⁵. Genetic studies have demonstrated that 2,4-diacetylphloroglucinol (Phl), pyoluteorin (Plt), pyrrolnitrin (Prn), phenazines (Phz), and hydrogen cyanide (HCN) can contribute to disease suppression in pseudomonads^{15, 39}. Naturally occurring pseudomonads involved in suppressiveness to take-all of wheat or black root rot of tobacco are mostly Phl producers, whereas production of Phz, Plt, or Prn does not seem to play a role^{26, 62, 87, 94}. Phl producers are commonly found in the rhizosphere of important crops such as cucumber, tomato, tobacco, maize, and wheat^{88, 112}. Phl production occurs only in pseudomonads, as far as we know, and all these strains but one are also HCN producers¹¹². No case of synergism between antimicrobial metabolites is known, but biocontrol pseudomonads producing both Phl and HCN are more effective than those producing only HCN (our unpublished results). This is in accordance with the fact that both HCN and Phl productions contributed to suppression of *Thielaviopsis basicola*-mediated black root rot of tobacco by strain CHA0^{48, 109}. Interestingly, certain HCN-producing pseudomonads are in fact deleterious to the plant, but it is not known why certain HCN-producing pseudomonads are plant-beneficial and others plant-deleterious⁸⁶. The genome of the Phl-producing biocontrol pseudomonad Pf5, which is closely-related to strain CHA0⁸⁸, is in the process of being entirely sequenced (<http://www.wsu.edu/pseudomonas/status.htm>).

Here, Phl will be reviewed as a key example of biocontrol substance because much less is known on the other biocontrol secondary metabolites. Several biotic and abiotic factors modulate Phl synthesis. For instance, Plt and salicylic acid act on the *phlA-phlF* region and repress Phl synthesis in strain CHA0³⁹. The toxin fusaric acid produced by the fungal pathogen

Fusarium oxysporum has the same negative effect as salicylic acid on Phl production, which allows the pathogen to protect itself against the biocontrol agent³⁰. The genotype, the age of the plant host, and the presence of saprophytic *Fusarium* modulate *phl* expression, whereas root infection by *Pythium* stimulates *phl* expression^{76, 77}. Interestingly, Phl itself boosts *phl* expression in several Phl-producing pseudomonads, by dissociating the PhlF repressor. This was observed in vitro and on the roots³⁹. Abiotic factors (e.g., Zn^{2+} , Cu^{2+} , $\text{NH}_4\text{Mo}^{2+}$, Fe^{3+} , oxygen tension, and carbon sources) influence also the production of Phl in strain CHA0²⁹. Moreover, addition of Zn^{2+} and $\text{NH}_4\text{Mo}^{2+}$ increased the efficacy of CHA0 against *Fusarium* and phytoparasitic nematodes^{30, 42}. Phl-producing pseudomonads reduce disease incidence caused by bacteria, fungi, and nematodes in several host plants. This correlates with the fact that Phl is toxic for bacteria, fungi, nematodes, anthelmintic worms, and plants under in vitro conditions³⁹. The mode of action of Phl is not well known. In the case of certain fungi, one hypothesis is that Phl could act on melanin synthesis. Indeed, strain CHA0 (but not a Phl-minus mutant) inhibits the formation of melanin in *T. basicola* and *Gaeumannomyces graminis* at concentrations which still allow the growth of the hyaline hyphae (our unpublished results). This observation is in accordance with the fact that phloroglucinol is a potent inhibitor of the conversion of dopa to dopachrome, a key step in the biosynthesis of melanin pigments¹⁹. The loss or inhibition of melanin production is associated with the loss of pathogenicity in several fungi^{10, 44}. Sensitivity levels to Phl vary about 100 fold among fungal species and isolates of the same species³⁰. The amount of Phl is estimated to be 3 ppm at the infection sites³⁹. This concentration is enough to slow down the growth of many pathogens during the infection process and gives time to the host plant to activate its defence mechanisms. Indeed, pathogenic strains whose growth was inhibited in vitro at least at 25% by 3 ppm of Phl could be controlled by Phl-producing pseudomonads during the take-all infection process, whereas tolerant strains were not controlled⁶⁴.

Antagonistic effects on phytopathogens can also be mediated by extracellular lytic enzymes, and many biocontrol pseudomonads produce such enzymes⁹². However, their effects on growth of the pathogen and the resulting contribution of pathogen inhibition to plant protection have received little attention in comparison to work done on other bacterial taxa. Lytic enzymes can also act at other levels, for example, by interfering with pathogenicity (or virulence) factors of the pathogens, or with the signal molecules that trigger production of secondary metabolites involved in pathogenicity. For instance, tomato plants can be protected against wilt caused by *F. oxysporum* f. sp. *lycopersici* by inoculation with a non-*Pseudomonas* strain able to degrade a virulence factor (fusaric acid) produced by the fungus⁹⁸, and whether this type of mechanism applies for biocontrol pseudomonads needs to be assessed. In addition, several

research groups have assessed the possibility to reduce the incidence of bacterial diseases by blocking quorum sensing cell–cell communication between pathogenic bacteria^{11, 69, 104}. Indeed, several non-pathogenic bacteria (including pseudomonads) can degrade *N*-acyl-homoserine lactones (AHLs) quorum sensing signals¹⁰⁴. The pathogens *Agrobacterium tumefaciens* and *Erwinia carotovora* were much less pathogenic to tomato roots and potato tubers respectively in the presence of a *Pseudomonas* genetically modified to degrade AHLs⁶⁹.

3.2. Biocontrol Interactions via the Plant

In addition to a direct effect on phytopathogens, some *Pseudomonas* strains can contribute to disease suppression via a positive, direct effect on the plant. In the case of biocontrol pseudomonads, this effect corresponds mainly to the induction of resistance⁸³, which does not require a direct contact between the biocontrol pseudomonad and the pathogen⁵⁹. This systemic resistance is long-lasting and protects plants against a broad range of pathogens and parasites, and in some cases against insects^{15, 116}. By inducing resistance, root-colonizing biocontrol pseudomonads favour optimal functioning of the plant, including photosynthesis in shoots. Part of the assimilates will be translocated to the roots⁵⁴ and released into soil as root exudates, which these biocontrol pseudomonads will use as substrates for growth⁴⁹. Therefore, the relationship between resistance-inducing pseudomonads and the plant appears to be an associative symbiosis.

Two physiologically different responses of the plant, termed induced systemic resistance (ISR) and systemic acquired resistance (SAR), may be involved when resistance is induced by root-colonizing bacteria. The ISR response requires production of jasmonic acid and ethylene by the plant, as shown with *Arabidopsis* mutants not responding to either jasmonic acid or ethylene⁸³. During ISR, priming of the expression of plant genes involved in resistance allows the plant to react more efficiently to an invading pathogen^{15, 83}. Cell surface constituents of pseudomonads, such as lipopolysaccharides, can activate ISR⁸³. SAR was discovered after infection of leaves with an agent (virus, bacterium, fungus) causing small necroses⁴⁵. The onset of SAR is associated with the appearance of pathogenesis-related (PR) proteins in intercellular fluids at the infection site and in protected parts of the plant^{45, 59}. The presence of salicylic acid is necessary to activate PR-related genes, but salicylic acid is not the systemic signal, as shown using transgenic tobacco plants able to degrade salicylic acid¹⁰⁷. Several metabolites (e.g., salicylic acid, pyoverdine, some derivatives of phenazines) produced by root-colonizing pseudomonads have been proposed to elicit SAR^{2, 59, 60}. Although they are often observed in response to different strains, SAR and ISR do not mutually exclude each other. For example, strain CHA0 induces SAR in tobacco against

the tobacco necrosis virus, and ISR in *Arabidopsis* against the fungus *Peronospora parasitica*^{39, 59}. ISR and SAR can take place simultaneously in *Arabidopsis*, leading to enhanced effects against the leaf pathogen *Pseudomonas syringae*¹⁰⁶. Evidence for the importance of induced resistance has been obtained also in field experiments¹¹⁷. Yet, it is unknown if ISR and SAR play a role in agrosystems in the absence of inoculation, or in natural ecosystems, as induced resistance is likely to represent a significant cost for the plant. Biocontrol pseudomonads isolated from a suppressive soil can induce plant resistance following inoculation⁵⁹, but no report exists on the existence of disease-suppressive soils where ISR or SAR actually takes place.

Apart from induced resistance, *Pseudomonas* strains may also have a positive, direct effect on the plant by altering hormonal balance. This mechanism is not entirely disconnected from induced resistance, as low concentration of plant ethylene is necessary to induce ISR. A high ethylene concentration increases plant susceptibility to disease¹. Several pseudomonads are able to cleave enzymatically the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) into ammonium (i.e., a nitrogen source for these pseudomonads) and α -ketobutyrate^{81, 111}. This ACC deaminase activity reduces disease incidence in some but not all pathosystems¹¹¹. In the absence of pathogens, pseudomonads with ACC deaminase activity were found to promote root growth and help the plant resist heavy metal stress³⁷. In addition, many biocontrol bacteria including pseudomonads have the ability to synthesize the plant hormone indole-3-acetic acid (IAA)⁷⁹. This may seem surprising, as IAA is known to down-regulate plant defence responses¹¹⁵. In non-pathogenic bacteria, IAA is involved in development of the host root system⁷⁹. In this context, enhanced IAA production by a genetically modified derivative of strain CHA0 had no influence on the ability to protect cucumber against *Pythium* in natural soil, but it resulted in increased fresh root weight in the absence of the pathogen⁶. In the model proposed by Glick³⁷, it is proposed that IAA production and ACC deaminase activity act together to promote root elongation. Therefore, these findings suggest overall that certain bacteria can help the plant fine-tune its hormonal balance in response to particular environmental conditions. In conclusion, it appears that biocontrol pseudomonads can act indirectly on the pathogens in a variety of ways, noticeably by inducing defence mechanism, reducing susceptibility factors, and/or promoting plant growth and strength.

3.3. Interactions with Non-target Microorganisms

Interactions of biocontrol pseudomonads with non-target microorganisms can be expected based on competition phenomena and the fact that many biocontrol pseudomonads produce one or several toxic metabolites. First,

a biocontrol pseudomonad may be expected to interact with other biocontrol microorganisms, including biocontrol pseudomonads. Indeed, the percentage of the latter amongst the total aerobic bacteria or the total fluorescent pseudomonads increases inside disease lesions, promoting interactions between biocontrol pseudomonads, and this increase is associated with disease control^{4, 14, 27, 87}. Accordingly, the percentage of Phl-producing isolates amongst the total culturable fluorescent pseudomonads on infected roots was higher in suppressive soils compared with conducive soils. This percentage was 31% for Swiss soils naturally suppressive to black root rot of tobacco (vs 7% in conducive soils⁸⁷) and 13% for Dutch soils suppressive to wheat take-all (vs 2% or less in conducive soils²⁷). These results could be due to the inhibition of rhizosphere microorganisms, including some that compete with the biocontrol agent (and/or the pathogen) for root exudates⁴⁷, but the ability to synthesize antimicrobial secondary metabolites did not always confer a selective advantage to individual *P. fluorescens* inoculants for plant colonization^{13, 20, 63}. Another possible explanation for the higher proportion of Phl-producing pseudomonads during disease control could be Phl-mediated autoinduction, as the presence of Phl results in higher *phl* expression³⁹. Indeed, Phl produced by CHA0 is perceived as a signal that boosts Phl production in other biocontrol pseudomonads, and vice versa (M. Maurhofer, E. Baehler and C. Keel, personal communication). Healthy and diseased roots are colonized by a functional community of Phl producers, which may differ from one another in their *phlD* biosynthetic allele^{61, 87}. The polymorphism of *phlD* was found to be a good marker for catabolic and other phenotypic diversity^{50, 82, 88, 112}. This high diversity allows a better adaptation to the changing environmental conditions on root during the disease control process in suppressive soils. We can therefore speculate that the better-adapted genotypes multiply until their populations reach the level necessary to stimulate Phl synthesis, which in turn will induce Phl production in the other strains. However, the higher proportion of Phl-producing cells in the disease lesions may also result from the effect of a third party, for example bacteria like *Chryseobacterium*, which seems to inhibit most microorganisms except Phl producers and the pathogen at the lesion microsite⁶⁵.

Second, a biocontrol pseudomonad may interact with microorganisms that are neither biocontrol agents nor pathogens. It does not mean that such interactions are necessarily irrelevant in terms of biocontrol, as for instance the latter microorganisms may have direct beneficial effects on the plant (e.g., plant microsymbionts). Yet, they have mostly been studied in a context of inoculation with a biocontrol pseudomonad, the focus being on the impact on non-target microbiota. The rationale behind this approach is that the toxicity of many biocontrol metabolites produced by *Pseudomonas* inoculants is rather broad, raising the possibility of negative side effects in the agro-ecosystem.

Information about potential effects on non-target organisms is necessary for inoculant registration^{16, 70}. The impact of a biocontrol pseudomonad is often assessed in the absence of the pathogen for which the inoculant was developed, so that potential negative effects are not masked by positive, biocontrol effects. Non-target microorganisms include resident fluorescent pseudomonads, and in soils that are not disease-suppressive the majority of them do not have biocontrol properties. The impact of biocontrol *Pseudomonas* inoculants on resident fluorescent pseudomonads can be quite extensive^{24, 68, 71}. This impact results mostly from competition phenomena, and resident strains capable of using carbon substrates not assimilated by the inoculant are favoured over other resident strains. Indeed, most resident pseudomonads are rather insensitive to the antimicrobial metabolites (e.g., Phl, Plt) produced by biocontrol strains^{68, 71}. The impact on plant microsymbionts, especially rhizobia and mycorrhizal fungi, is of major interest because of their significance for plant growth and soil fertility. Many rhizobial strains are inhibited by biocontrol metabolites produced by pseudomonads (e.g., Phl¹¹⁰). The effect of biocontrol pseudomonads on rhizobia was of little importance in all systems tested^{23, 34, 75, 108}, and in one case a slightly positive effect was recorded²³. In a field experiment, nodulation of soybean by a *Bradyrhizobium japonicum* inoculant was increased following inoculation with a marked derivative of Phl-producer *P. fluorescens* F113, despite Phl sensitivity of the microsymbiont in vitro¹⁸. The positive effect of the biocontrol pseudomonad resulted probably from the inhibition of soil microorganisms that were limiting nodulation by *B. japonicum*, and it illustrates how the potential for a negative effect inferred from in vitro experiments can transform into a net positive effect within the microbial community in situ. In one study, the effect of *P. fluorescens* F113, which was inoculated to sugarbeet seeds, was assessed on resident *Rhizobium leguminosarum* nodulating a red clover rotation crop^{67, 110}. The inoculant did not influence the extent of root nodulation on the red clover seeded as subsequent crop or the functioning of the *Rhizobium*–clover symbiosis⁶⁷, but rhizobial diversity was reduced¹¹⁰. This work is the first documented case of a residual impact of a biocontrol inoculant. Data pointed to Phl production as a possible explanation for the impact. Other hypotheses were put forward but could not be addressed in that work, for example, the role of the bacteriocin *small*, which inhibits certain rhizobia and is produced as quorum sensing signal by strain F113⁵². Several *Pseudomonas* strains are helpers of ectomycorrhizal formation³³, but whether they also display biocontrol properties is not clear. The impact of biocontrol pseudomonads on endomycorrhizae seems of small magnitude, if any^{3, 31, 34, 89}. Positive interactions have even been found. In the tomato rhizosphere for instance, *P. fluorescens* F113 enhanced mycelial growth of *Glomus mosseae* and root mycorrhization, whereas *G. mosseae* increased the population of CHA0³¹. Phl seems to have no effect on the mycorrhizal symbiosis^{3, 31}.

In addition to specific microbial groups, the impact of biocontrol pseudomonads has also been assessed at higher ecological levels, that is, at the level of the total bacterial or fungal community. Despite the fact that many indigenous microorganisms are sensitive in vitro to the antimicrobial metabolites produced by biocontrol pseudomonads^{35, 47, 72}, no long-lasting detrimental effect of biocontrol pseudomonads was found in natural soil^{24, 35, 36, 47, 96}. The transient effects observed were smaller than for example, the effect of plant age or of growing a crop repeatedly^{35, 72}.

4. CONCLUSION

Biocontrol pseudomonads represent prominent scientific models in various fields of microbiology, ranging from the regulation of secondary metabolism to the mechanisms involved in microbial interactions. They owe this status to their importance for plant health, which has been extensively documented for individual *Pseudomonas* strains. Their phytobeneficial effects have also been assessed at the level of the functional community of biocontrol pseudomonads in disease-suppressive soils, where these effects represent an emerging ecosystemic property. Biocontrol pseudomonads are involved in multiple interactions with the pathogen(s), the plant host, and non-pathogenic microorganisms. Many interactions with the latter are likely to contribute, indirectly, to the resulting beneficial effect(s) of biocontrol pseudomonads on the plant, and understanding them remains a major scientific challenge.

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THE PATHOGENIC LIFESTYLE OF *PSEUDOMONAS AERUGINOSA* IN MODEL SYSTEMS OF VIRULENCE

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1. INTRODUCTION

It is a constant challenge for the microbiologist not to let a human's-eye view of the world occlude a bacterium's-eye view⁷¹. From a human perspective, *Pseudomonas aeruginosa* seizes the opportunity to proliferate and cause acute disease¹⁰. Any breach of external barriers provides such an opportunity and includes tissue damage (burned skin or a scratched cornea) and procedures that allow contamination of the blood or urinary tract. Inborn mutation of the human *CFTR* gene results in a lung environment where *P. aeruginosa* can persist and cause chronic disease. Other organisms, when weakened, are also at risk. *P. aeruginosa* can kill waterlogged plants³⁰ and overcrowded grasshoppers¹². Because of its potential for harm, *P. aeruginosa* is classified as an opportunistic pathogen (Figure 1).

An alternative viewpoint, however, is suggested by the observation in 1863 that the presence of moderate amounts of blue pus in human wounds correlated with a better prognosis. *P. aeruginosa* was isolated from such

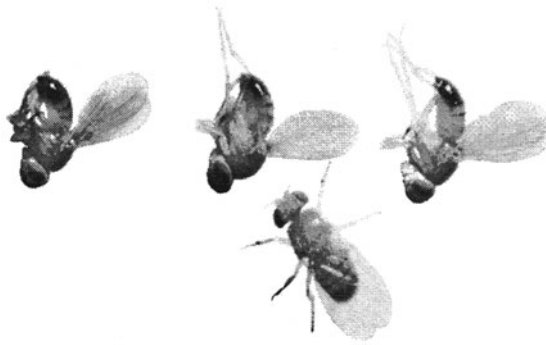


Figure 1. *P. aeruginosa* can be lethal to the fruitfly *Drosophila melanogaster*^{11, 17, 27, 34, 70, 123}.

wounds in 1882^{121, 134} and the original observation is probably explained by the fact that this bacterium secretes a variety of antibiotic agents including the blue phenazine pigment pyocyanin^{13, 50, 72, 75, 105, 121, 134}. *P. aeruginosa* kept at bay other bacteria that were more likely to cause a fatal infection. From this perspective, the constellation of traits that define *P. aeruginosa* have been shaped over evolutionary time not by opportunities to cause human disease but rather by the demands of the microbial world where the distinction between friend, foe, and food is not so certain. The most complete understanding of *P. aeruginosa* then, encompasses knowledge of its interactions with its soil and water co-inhabitants including plants, insects, nematodes, protozoa, and fungi.

2. PLANTS

Many members of the genus *Pseudomonas* interact with plants. *Pseudomonas syringae* is a model bacterial pathogen of plants while strains of *Pseudomonas fluorescens* and *Pseudomonas putida* promote plant growth by inhibiting the growth of fungal pathogens⁹⁴. Less is known about the spectrum of interactions between *P. aeruginosa* and plants. Although this bacterium thrives in moist conditions in general, it is also commonly recovered from plants¹⁰. In one study, *P. aeruginosa* was cultured from 80% of the tomatoes delivered to hospital kitchens. Adding further complexity, strains of *P. aeruginosa* differ in part due to the presence of islands of chromosomal genes shared with bacteria that interact intimately with plants^{69, 110}.

2.1. An Agricultural Pest

A bacterial leaf spot and soft-rot disease that caused economically significant damage was described in crop plants including tobacco, lettuce, and sugar cane. The bacterial agent of the disease was tentatively identified as *P. aeruginosa*³⁰. Supporting this identification, *P. aeruginosa* isolates from soil, plants, and animals (including human clinical isolates) were shown to cause the leaf spot symptoms in tobacco and lettuce. They also could produce soft-rot in lettuce, cucumber, potato, and onion that was similar to that caused by another bacterium, the plant pathogen *Erwinia carotovora*. As is often the case for *P. aeruginosa*, pigment production distinguished strains in a useful way: Strains producing more pigment were more invasive in plants³⁰.

In a subsequent study in which antibodies were generated against bacterial pathogens of plants, *P. aeruginosa* was found to be unique²⁹. The bacterium grew within and killed not only plants, but also rabbits, mice, and guinea pigs. Nevertheless, *P. aeruginosa* was considered not to be an aggressive pathogen. Injection of relatively large doses was required for a fatal infection in laboratory animals. In crop plants, systemic infection was associated with unfavorable weather conditions including frost, high temperature and humidity, and growth conditions in general that led to water soaking. Under these conditions, the less actively growing lower leaves of the plant were more sensitive to damage while being readily exposed to the reservoir of *P. aeruginosa* in the soil³⁰. Although environmental conditions can favor growth of *P. aeruginosa* at the expense of a plant host, this outcome may be the exception. Disease, however, is more readily noticed than health.

2.2. An *Arabidopsis* Model

The mustard weed *Arabidopsis thaliana* was successfully used as a model host for the plant pathogen *P. syringae*¹²⁵. A collection of *P. aeruginosa* strains was therefore screened for the ability to cause disease symptoms when injected into leaves of intact *Arabidopsis* plants⁹⁹. *P. aeruginosa* strain PA14, an isolate from a burn patient, caused severe symptoms in *Arabidopsis* ecotype Llagostera^{51, 93, 98, 99}. Leaves were injected with 10^3 PA14 cells and the plants were incubated at 28°–30°C. After 5 days, PA14 had caused severe soft-rot throughout the leaf and had reached a density in the leaf of approximately 10^9 colony forming units (CFU) per gram (Figure 2). Similarly, a density of up to 10^9 *P. aeruginosa* CFU per ml is reached in the sputum of cystic fibrosis patients¹⁰⁹. A detailed analysis using detached *Arabidopsis* leaves revealed that PA14 attached by one pole to plant cell walls in the infected tissue, forming holes the size of the bacterium. The entire surface of such plant cells became

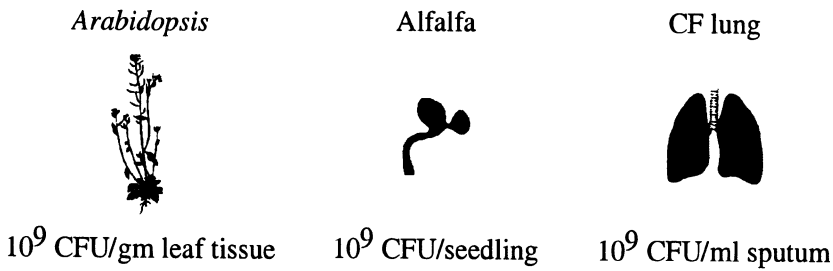


Figure 2. Growth of *P. aeruginosa* within plants and humans (the indicated bacterial densities are associated with soft-rot in *Arabidopsis* and alfalfa and with chronic lung infections in cystic fibrosis patients).

convoluted presumably due to more diffuse enzymatic degradation. Plant cell lysis followed, with bacteria moving from cell to cell along (rather than within) small leaf veins until the entire leaf was digested. Bacteria were not seen within apparently healthy cells. For leaves dipped in a bacterial suspension, stomata provided natural openings for *P. aeruginosa* infection.

2.2.1. A Leaf Wounding Assay as a Screen. The ability of *P. aeruginosa* to kill plants and animals was linked to its ability to grow within these hosts^{29, 30}. This association between virulence and growth held true not only in the *Arabidopsis* leaf wounding assay but also in a mouse burn assay using a bacterial dose of 5×10^3 or 5×10^5 cells. *P. aeruginosa* PA14 proliferated within the wounded tissue in both assays, ultimately causing a systemic infection^{98, 99}. *P. aeruginosa* strain PAO1, the human wound isolate whose genome has been sequenced¹¹⁵, was also virulent in both assays but caused fewer fatal infections in mice than PA14.

Engineered mutations in PA14 that inactivated *toxA*, *plcS*, or *gacA* impaired growth in each model host with a corresponding reduction in soft-rot in plants and mortality in mice⁹⁹ (Table 1). Five days after infection of *Arabidopsis* leaves, each mutant was at a lower cell density than PA14, and the CFU of the *gacA* mutant had dropped (after an initial increase) to approximately the level of the inoculum. The *plcS* gene encodes the secreted hemolytic phospholipase C, and *gacA* encodes the response regulator of a two-component regulatory system that overlaps with the quorum sensing system to control numerous virulence factors¹⁰². The *toxA* mutant lacks exotoxin A, a secreted virulence factor that inhibits eukaryotic protein synthesis. The function of exotoxin A has not been documented in plants. Nevertheless, it is noteworthy as a secreted enzyme, not directly involved in tissue degradation, that is predicted to perturb the physiology of both plant and animal cells.

A screen of 2,500 transposon insertion mutants of strain PA14 identified nine mutants that grew like the wild-type strain in rich and minimal medium,

Table 1. Selected *P. aeruginosa* PA14 virulence-impaired mutants.

Gene mutated ^a	<i>Arabidopsis</i> soft-rot ^b	<i>C. elegans</i> fast killing ^c	<i>C. elegans</i> slow killing ^c	LD ₅₀ in <i>G. mellonella</i> ^d	% Mouse mortality ^e
WT	+++	+++	+++	1	100
<i>toxA</i>	(++) ^f	+++	++	2	40
<i>plcS</i>	(++)	+++	+++	20	40
<i>gacA</i>	(+)	+++	(+)	10–100	50
<i>gacS</i>	+	+++	(+)	NT	50
<i>lasR</i> ^g	++	+++	(+)	5	50
<i>mvfR</i>	(++)	+	++	3	56
<i>pqsB</i>	++	(+)	+++	100	63
<i>mdoH</i>	–	(+)	less	10	0
<i>phzB</i>	++	(+)	+++	10–60	18

^aThe parental wild-type (WT) strain is PA14; the mutants are described in refs 14, 57, 78, 98, 116, 118, and 119. Gene designations, some updated in a review of PA14 mutants¹¹⁶, refer to annotations from strain PAO1 where *plcS* has been renamed *plcH*, and gene PA0997 has been designated *pqsB*. The *toxA*, *plcS*, and *gacA* mutations were engineered; all others are insertions of *TnphoA*. A *gacA* *TnphoA* insertion mutant was also identified in two screens, one using the lettuce leaf assay and one using the nematode slow-killing assay.

^bSeverity of soft-rot symptoms, from severe (+++) to none (–).

^cVirulence, measured as the time to kill 50% of the worms assayed, from highly virulent (+++) to severely impaired virulence (+); the *mdoH* mutant was less virulent in nematode slow killing, but the data was not shown.

^dThe number of bacterial cells required to kill 50% of larvae by 60 hr; one mutant was not tested (NT).

^ePercentage of mice killed by 7 days in the mouse burn assay after infection with 5×10^5 bacterial cells.

^fParentheses distinguish strains that were identified using the model system for that column; such strains were subsequently tested in other models.

^gThis strain may have an additional mutation¹¹⁹; It was noted to overproduce¹¹⁹ or under-produce⁹⁶ pyocyanin, and *lasR* expressed from a plasmid complemented the virulence phenotypes but not the pyocyanin phenotypes.

but grew less well in lettuce causing less pronounced soft-rot^{98, 100}. Detached lettuce leaves were used to facilitate screening, and virulence defects were subsequently confirmed in *Arabidopsis* leaves. All nine mutants were also impaired in virulence in the mouse burn assay. One strain carried a mutation in *mvfR* (Table 1), a gene later shown to be required for biosynthesis of the *Pseudomonas* quinolone signal (PQS), an extracellular signal that interacts with quorum sensing^{15, 26, 42, 91}. PQS production is induced in vitro by growth conditions that induce lipopolysaccharide modifications typical of *P. aeruginosa* chronically infecting the lungs of cystic fibrosis patients⁴⁷, and PQS can be recovered from the lungs of such patients¹⁹. In one PAO1 mutant, PQS overproduction correlated with cell-density dependent autolysis²⁶. Since autolysis is a common phenotype in *P. aeruginosa* isolates, PQS may play a fundamental role in tuning cellular physiology.

2.2.2. *Arabidopsis* Immunity. *P. aeruginosa* PA14 caused varying degrees of soft-rot in different *Arabidopsis* ecotypes, and the severity of symptoms

correlated with the degree of bacterial growth within the plant⁹⁹. Some ecotypes restricted *P. aeruginosa* growth enough to prevent observable disease, as is also the case for *P. syringae*. Suggesting an additional mechanism of resistance, PA14 cells adhered over five fold less efficiently to leaves of a resistant *Arabidopsis* plant than to leaves of a sensitive ecotype⁹³. The biology of resistance is steadily being revealed using the genetic tools available for plant model hosts. A transgenic tobacco plant expressing the *P. putida* gene for catabolism of salicylic acid provided key support for the role of this compound as an endogenous signal that triggers plant defenses⁴⁰. An *Arabidopsis* mutant blocked in this signaling system was shown to have enhanced susceptibility to *P. aeruginosa* PA14 and to other bacterial and fungal pathogens of plants¹²⁵. Plant defenses share elements with the innate immune response in animals, including secretion of antimicrobial peptides and molecular recognition of microbial cell components by receptors with leucine-rich repeats²⁴.

2.3. An Alfalfa Seedling Model

In chronic *P. aeruginosa* infections in cystic fibrosis patients, there is strong selection for strains that carry a mutation causing overproduction of the bacterial exopolysaccharide alginate^{45, 110}. *P. aeruginosa* strain FRD1 is one such mucoid isolate. Although the non-mucoid strain PAO1 persists in a rat lung model of chronic infection, FRD1 is quickly eliminated¹⁰⁸. Both strains, however, caused readily visible soft-rot by 6 days after injection into alfalfa seedlings incubated at room temperature¹⁰⁸. PAO1 reached a density of 2×10^9 CFU per seedling (Figure 2) while due to the physiological burden of alginate overproduction, FRD1 grew to a lower density within the plant and caused less severe soft-rot. Nevertheless, mucoidy of FRD1 was stable during growth within seedlings (in contrast to mucoidy in a PAO1 derivative with an engineered mutation).

Strain FRD1 is mucoid because of a mutation that inactivates the anti-sigma factor Muca. This results in increased expression of the extreme stress sigma factor AlgT (also known as AlgU) and the consequent over-expression of alginate biosynthetic genes. As expected, inactivation of *algT* suppressed mucoidy in FRD1. The FRD1 *algT* mutant was also less virulent in alfalfa seedlings (as was an FRD1 mutant defective in the *rhl* quorum sensing system). Using a defined dose of bacteria, fewer seedlings showed symptoms after infection with either mutant compared to infection with the parental strain FRD1¹⁰⁸. Having a variety of models to study the physiological role of AlgT is particularly valuable given the complexity of the stress response associated with mucoidy³⁷. Mutation of *algT* can have different effects on virulence depending on the strain of *P. aeruginosa* and on the model host^{108, 135}. Similarly, engineered mutations in strain PA14 revealed that suppression of

mucooidy had different effects on virulence in *Arabidopsis* and mouse models of infection¹³³.

3. INSECTS

For insects that have a diet rich in microorganisms, it is a particular challenge not to be overwhelmed by their food. If fed in sufficient quantities to house fly larvae, *P. aeruginosa* can persist through metamorphosis, ending up within the adult fly⁵. Bacterial persistence favors dissemination by insect vectors. Plant pathogens in the genus *Erwinia* can be transmitted to plants by fruitflies, and the bacteria are specifically recognized but not eliminated by the fly innate immune system^{7, 122}.

3.1. A Laboratory Pest

P. aeruginosa was identified as the agent of a deadly disease in laboratory colonies of grasshoppers^{12, 114}. The source of the bacterium was traced to grasshopper eggs collected in the wild. The humid growing conditions in the laboratory probably facilitated both the infection of newly hatched grasshoppers as well as the subsequent food and water contamination that spread the disease. Fatal infections were reproduced when *P. aeruginosa* was introduced into grasshoppers by injection or feeding, with the bacterium growing within and extensively digesting the insect. In contrast, introduction by either route of a large dose of the grasshopper's normal bacterial flora was harmless. Injection of 10–20 *P. aeruginosa* cells was sufficient to kill 50% of the treated grasshoppers. After infection and incubation at 30°C, the *P. aeruginosa* CFU within the insects initially dropped slightly and then recovered, increasing with a doubling time of 79 min. Grasshoppers (weighing on average 1 g) generally died 48 hr after infection. At death, they contained approximately 10¹⁰ *P. aeruginosa* CFU (Figure 3). Shortly before death, they appeared as active as uninfected insects even though they carried 10⁹ CFU. This linking of an insect disease to infection by a bacterium has an illustrious historical antecedent: The first proof that a disease in animals was caused by a microorganism was the demonstration in 1834 that silkworms were being killed by a fungus¹¹³.

3.2. A *Drosophila* Model

The fruitfly *Drosophila melanogaster* has been used as a model host to study infection by diverse pathogenic microorganisms^{28, 122} including bacteria such as *P. aeruginosa*¹¹. Unperturbed fruit flies can live for over 3 weeks in the laboratory. Flies wounded with a syringe needle dipped in a liquid culture of

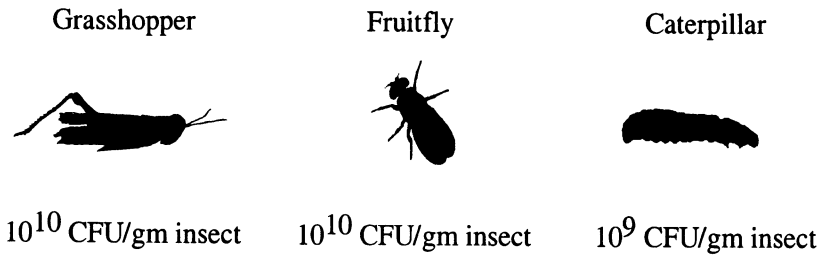


Figure 3. Growth of *P. aeruginosa* within insects (lethal bacterial densities are indicated).

P. aeruginosa PAO1 (providing a dose of 400–2,000 bacterial cells) generally died 24 hr after infection when incubated at 25°C²⁷. After an initial decrease in CFU, the bacterium grew within the fly, doubling every 78 min. At death, flies carried approximately 10^7 *P. aeruginosa* CFU, an amount roughly equivalent to that which is lethal to grasshoppers, adjusting for body weight (Figure 3). Flies appeared healthy until one hr before death. Other strains of *P. aeruginosa* were comparably lethal to fruit flies^{11, 34, 70, 123}. The initial lag in bacterial growth, also seen after direct injection of bacterial cells into the fly¹¹, suggests that early events in the infection may be particularly important in determining the outcome.

3.2.1. *A Drosophila Wounding Assay as a Screen.* A collection of 1,500 transposon insertion mutants of *P. aeruginosa* PAO1 was screened using the fruitfly as a model host²⁷. Individual fruit flies were infected by pricking and incubated at 25°C in wells of a 48-well tissue culture dish. Those mutants with the strongest impairment in fly killing had the compact colony morphology characteristic of strains lacking twitching motility, a form of surface motility mediated by Type IV pili⁸¹. Most of these mutants had an insertion in the *pil chp* gene cluster and exhibited a delay of 6 hr in killing 50% of the flies. The *pil chp* genes encode a chemotaxis-like signal transduction system that is required for twitching motility⁸¹. This signaling system appears to control the expression of as yet undetermined virulence factors because twitching motility itself was not required for full virulence in the fly²⁷. Unlike the virulence-impaired mutants in the plant and mouse model hosts, a *chpA* mutant grew within the fly at the same rate as the parental strain. Compared to flies infected with PAO1, those infected with a *chpA* mutant died later and carried approximately 10 fold more bacterial cells.

PAO1 and other *P. aeruginosa* strains kill various human cell types in tissue culture, including macrophages and epithelial cells^{34, 61}. A screen for transposon insertion mutants of strain PA103 that were non-cytotoxic towards epithelial cells identified genes in the *pil chp* cluster (*pilJ* and *chpA*), structural

genes for Type IV pilus biosynthesis, and Type III secretion genes⁶¹. The Type III secretion system is used to inject proteins into eukaryotic cells. In *P. aeruginosa* strain CHA, an isolate from a cystic fibrosis patient, a mutation was engineered that inactivated ExsA, a transcriptional activator of Type III secretion genes. This mutation also eliminated cytotoxicity²³. In the fruitfly wounding assay, this *exsA* mutant exhibited a delay of approximately six hr in killing 50% of the flies compared to the parental strain³⁴. The delay corresponded to an apparent lag in achieving exponential growth within the fly. Consistent with a role for Type III secretion in fly killing, the *exsA* gene was strongly induced in *P. aeruginosa* cells growing within the fly. Which substrate of this secretion system is involved in fly killing is not yet known, but strain CHA does not appear to carry the *exoU* gene encoding one such effector protein²³.

3.2.2. *A Drosophila Feeding Assay.* While the fly wounding assay may mimic the external tissue damage exploited by opportunistic bacterial pathogens, a feeding assay probably more closely reflects the way insects in the wild become infected⁷. The bacterium *Serratia marcescens*, an opportunistic pathogen, can cause disease in a variety of organisms including humans and is lethal when fed to fruit flies³⁸. In contrast, *P. aeruginosa* PAO1 was relatively benign when fed to fruit flies incubated at 25°C¹⁷. Only a minority of the flies was killed over the course of 2 weeks when a sucrose solution with 8×10^9 PAO1 cells was the sole food source. An engineered *qscR* mutant of PAO1, however, killed 50% of the flies by 5 days, and all of the flies by 12.5 days¹⁷. Flies died when they carried approximately 10^7 CFU of the *qscR* mutant as was the case with PAO1 in the feeding and wounding assays. QscR is a transcriptional repressor of LasI, one of the two quorum sensing signal generators. The *qscR* mutant therefore over expresses virulence factors controlled by quorum sensing. Compared to PAO1, for instance, it produces more pyocyanin, and produces the pigment at a lower cell density. The *qscR* mutant did not have a strong or reproducible phenotype in the fly wounding assay, and the cause of the hypervirulence phenotype in the fly feeding assay remains to be determined.

3.2.3. *Drosophila Immunity.* Studies of insect innate immunity have been particularly fruitful in revealing key aspects of the evolutionarily related mammalian innate immune system^{55, 63}. Studies with *Drosophila* led to the identification of the mammalian Toll-like receptor 4 (TLR4), crucial for the response to Gram-negative bacteria. In fruit flies, engineered mutations that enhance signaling by the Toll pathway provide partial resistance to killing by *P. aeruginosa*⁷⁰. Although the basis for this resistance is not yet known, the Toll pathway is activated by Gram-negative peptidoglycan⁷⁴. Screening randomly mutagenized flies might identify mutants even more resistant to *P. aeruginosa*.

In insects, microorganisms are eliminated in part by a battery of secreted antimicrobial peptides. *P. aeruginosa*, fed to fruitfly larvae or introduced into adults by pricking, triggered expression of the fly dipterin gene that encodes an antimicrobial peptide active against Gram-negative bacteria^{7, 34, 73}. Such fly defenses, however, were relatively ineffective against *P. aeruginosa* in the wounding assay: Wild-type flies were killed as fast as an immune deficient mutant fly lacking any known antimicrobial peptide¹²³. Constitutive expression of a single antimicrobial peptide in this mutant fly conferred complete protection against *Bacillus subtilis*, a bacterium that was lethal when there was a lag before induction of the immune response as there is in wild-type flies. In contrast, relatively little protection was provided against *Staphylococcus aureus*, a bacterium that can be a pathogen of humans. These results emphasize the correlation between bacterial resistance to antimicrobial peptides and the potential for virulence¹²³.

3.3. A *Galleria mellonella* Model

Larvae of the greater wax moth *Galleria mellonella* weigh on average 250 mg and in part because of their large size, these caterpillars are an attractive model host for *P. aeruginosa* infection^{57, 85, 97}. Injection of only one cell of *P. aeruginosa* PA14 into *G. mellonella* larvae can be lethal⁵⁷. Larvae injected with up to 10^4 PA14 cells and incubated at 25°C generally died 48 hr after infection. As with fruit flies, larvae did not show symptoms of infection until shortly before death at which point they contained approximately 10^9 *P. aeruginosa* CFU per gram (Figure 3). One striking symptom was a black color due to melanization, a phenomenon associated with activation of the prophenoloxidase component of the innate immune response⁹⁷. Most of the PA14 mutants that were less virulent in other model systems were also impaired in killing *G. mellonella* larvae as reflected by an increase in the number of bacterial cells required to kill 50% of the larvae by 60 hr (Table 1). Similarly, these mutants killed less than 100% of fruit flies (in contrast to PA14) in a wounding assay in which flies were infected with 10–100 bacterial cells⁷⁰.

3.3.1. A Caterpillar Wounding Assay as a Screen. A screen of 1,560 transposon insertion mutants of strain PA14 identified seven mutants impaired in killing *G. mellonella* caterpillars⁸⁵. One mutant had an insertion in *pscD*, a gene predicted to encode a structural component of the Type III secretion apparatus. PA14 strains were subsequently engineered with mutations inactivating individual substrates of the Type III secretion system including ExoT and the lipase cytotoxin ExoU¹⁰³. Although ExoY and ExoS are also known Type III effector proteins in *P. aeruginosa*, PA14 does not express ExoY and does not carry the *exoS* gene⁸⁵. Mutation of either *exoT* or *exoU* did not detectably impair virulence. In contrast, the *exoTexoU* double mutant was

impaired in caterpillar killing indicating that the effects of either virulence factor could mask the contribution of the other. The *pscD* mutant and the *exoTexoU* double mutant had equivalent virulence phenotypes (measured as the increase in the number of bacterial cells required to kill 50 % of the caterpillars by one day after infection: Over 40,000 cells for the mutants as compared to 1–10 cells for PA14). However, when virulence was assayed at 2–4 days, the *pscD* mutant was more impaired in caterpillar killing than the effector mutant. This suggests that additional as yet unknown Type III secretion substrates play a role later in infection. Based on the phenotypes of these PA14 mutants in a cytotoxicity assay using CHO cells, such yet to be discovered effectors play a role in mammalian pathogenesis as well⁸⁵.

4. NEMATODES

Soil-dwelling nematode worms such as *Caenorhabditis elegans* feed on bacteria. Like fruit flies, such nematodes are potentially exposed to all the armaments of soil microorganisms¹²⁹.

4.1. A *C. elegans* Model

Just as *P. aeruginosa* was identified as a contaminant that was killing laboratory colonies of grasshoppers¹², *P. fluorescens* was identified as a contaminant that had killed a laboratory stock of *C. elegans*²⁵. This observation led to one line of research using the nematode to study *P. aeruginosa* virulence. Nematodes can live for 2 weeks in the laboratory, and a variety of bacteria and yeasts when given as food shorten this life span^{1, 21, 32, 43, 68, 86, 117}. For those bacteria that cause human disease, some may be too specialized to affect nematodes: The opportunistic pathogen *Burkholderia pseudomallei* was lethal to *C. elegans* while the obligate pathogen *B. mallei* was harmless⁸⁸. Virulence towards nematodes, however, can be highly dependent on assay conditions. *E. coli* strain OP50 is the standard laboratory food source for the nematode. When OP50 was grown on brain heart infusion agar, it slowly killed *C. elegans*⁴³, and even when grown using standard worm culture conditions, it detectably shortened the life of the worm compared to dead OP50 cells as a food source⁴⁴.

4.1.1. Paralytic Killing of *C. elegans* as a Screen. *P. aeruginosa* PAO1 grown for 24 hr at 37°C on brain heart infusion agar rapidly killed *C. elegans*^{25, 41}. Nematodes placed on the bacterial lawn (briefly cooled to room temperature) began to be paralyzed within seconds, and all worms were generally killed within 4 hr. The toxic activity was diffusible, a lethal amount remaining in the agar after removal of bacteria that had been grown on a filter²⁵. Further

illustrating the influence of the bacterial growth medium, *P. aeruginosa* PA14 was harmless in the standard paralytic killing assay (using 1.5% agar), but behaved identically to PAO1 when grown within brain heart infusion top agar (0.7%).

A screen of 3,000 transposon insertion mutants of strain PAO1 identified 25 strains (excluding those forming small colonies because of slow growth) that were impaired in paralytic killing⁴¹. One such strain was avirulent and had an insertion in *hcnC* encoding a subunit of hydrogen cyanide synthase. Subsequent assays revealed that the efficiency of worm killing correlated with the amount of cyanide gas each of the mutants produced. This explained the initially perplexing observation that worm killing was almost completely eliminated if the Petri dish lids were left off after the bacterial lawn was grown for 24 hr. Chemically generated cyanide gas, in an amount predicted to be equivalent to that generated by PAO1, reproduced paralytic worm killing. A *proC* mutant of PAO1, even when not in direct contact with *C. elegans*, protected the worms from cyanide poisoning, presumably by the accumulation of a metabolite that acted as a sink for cyanide gas.

Although cyanide was necessary and sufficient for worm killing, those nine mutants most strongly impaired in virulence had a pleiotropic phenotype indicating that cyanide production is physiologically complex⁴¹. The *hcnC* mutant overproduced pyocyanin, and expressing the *hcn* genes on a plasmid complemented this phenotype. The other less virulent mutants also had altered levels of secreted factors including pyocyanin. Similarly, in a separate collection of insertion mutants with visibly reduced pyocyanin production, many of the strains were impaired in worm killing⁴². As expected, this pleiotropy in some strains was explained by a mutation affecting the *las* or *rhl* quorum sensing systems that control production of both cyanide and pyocyanin. Other strains had a mutation in the gene cluster PA0996-PA1003 (including *mvfR* and *phnAB*), and this cluster was shown to be required for biosynthesis of the *Pseudomonas* quinolone signal that interacts with quorum sensing^{26, 42}.

Mutagenized worms were screened for resistance to paralytic killing by *P. fluorescens* strain SE59 that had been isolated as a laboratory contaminant toxic to *C. elegans*²⁵. Two worm mutants were identified, and both had a loss-of-function mutation in the *egl-9* gene required for normal egg laying. Mutation of *egl-9* conferred complete resistance to *P. aeruginosa* PAO1 and enhanced resistance to cyanide gas. In a separate line of research, Egl-9 and its human homologs were shown to play a key role in the response to hypoxia^{31, 107}. In humans, this physiological response can have negative consequences in cancer and chronic lung disease, but can also protect against tissue damage associated with heart attacks and strokes. Egl-9, the oxygen sensor in this response, is a dioxygenase whose activity is proportional to the level of ambient oxygen. A putative homolog of *egl-9* exists in some bacteria³ including *P. aeruginosa* PAO1 (gene PA0310).

4.1.2. Fast Killing of *C. elegans* as a Screen. *P. aeruginosa* PA14 grown on high osmolarity peptone glycerol sorbitol agar for 24 hr at 37 °C followed by 8–12 hr at room temperature was lethal to worms by a process termed fast killing^{78, 118}. Nematodes placed on the bacterial lawn died in 4–24 hr by a diffusible toxic activity. *P. fluorescens* strains 2–79 and WCS365 were just as lethal¹¹⁸. Unlike paralytic killing by PAO1, for fast killing the LasR regulatory component of quorum sensing was not required (Table 1), L4 larval stage worms were much more susceptible than adults, and *egl-9* mutant worms were not resistant^{77, 78, 118, 119}. For three other *C. elegans* mutants, relative resistance to oxidative stress correlated with resistance to fast killing⁷⁸.

A screen of 3,300 transposon insertion mutants of strain PA14 identified six mutants that were impaired in fast killing and that grew at a rate comparable to PA14 on rich and minimal medium⁷⁸. One of these strains, an *mdoH* mutant, was less efficient at worm killing and had a particularly strong phenotype in plants and mice: It caused no symptoms in plant leaves and no mortality in mice (Table 1). MdoH is required in other bacteria for the synthesis of osmoregulated periplasmic oligosaccharides, and *mdoH* mutants have a pleiotropic phenotype that includes virulence defects⁸⁹. Another fast-killing impaired PA14 mutant with a pleiotropic phenotype had an insertion in *pqsB* (Table 1), a gene involved in PQS biosynthesis^{26, 42}. Two independently generated mutants had identical insertions in a gene that is homologous to two PAO1 genes, *phzB1* and *phzB2* (Table 1). In PAO1, these two genes are part of two nearly identical operons encoding enzymes for phenazine biosynthesis⁸². PhzB may be involved in maintaining the physical association of these enzymes in a complex⁸³. A *C. elegans* mutant that lacked an efflux pump providing resistance to exogenous toxins was highly sensitive to fast killing, but was not killed by the *phzB* mutant⁷⁸. Phenazines alone were not sufficient for fast killing, since some of the other virulence impaired mutants did not appear to have a reduced pigment phenotype and produced a wild-type level of pyocyanin.

4.1.3. Slow Killing of *C. elegans* as a Screen. *P. aeruginosa* PA14 grown on standard worm growth medium (or a minimal medium) for 24 hr at 37°C followed by 8–24 hr at room temperature was lethal to worms by a process termed slow killing¹¹⁹. Nematodes placed on the bacterial lawn died in 2.5–3 days. In contrast to fast killing, slow killing required live bacteria in contact with the worms, and correlated with bacterial proliferation in the worm gut. The longer the worms were on the PA14 lawn, the less efficiently transfer to a lawn of *E. coli* rescued them. A screen of 2,400 transposon insertion mutants of strain PA14 identified eight mutants impaired in slow killing, and that grew like the parental strain on minimal medium¹¹⁹. Among the identified genes were *lasR*, *gacA*, and *gacS*, global regulatory genes required for virulence in other model hosts but not for fast killing of nematodes by PA14 (Table 1). The mechanism of slow killing remains unknown.

4.1.4. *C. elegans* Immunity. The nematode must limit microbial proliferation in the standard array of body cavities, but the mechanisms involved are less well characterized than those for insects^{32, 79, 95, 117}. Two *C. elegans* mutants were isolated that had enhanced susceptibility to slow killing by *P. aeruginosa* PA14⁶⁴. These mutants were also relatively sensitive to the Gram-positive pathogen *Enterococcus faecalis*. The mutated worm genes encoded components of a mitogen-activated protein kinase (MAP kinase) signaling pathway, a conserved element of innate immunity in plants, animals, and insects^{4, 64}.

5. PROTOZOA

Amoebae eat soil bacteria¹⁰¹, and are themselves prey for nematodes⁶². Selection in microorganisms for surviving predation by amoebae may have contributed to the evolution of virulence towards animals³³. A particularly direct link may exist for bacteria and fungi that are intracellular pathogens and that survive engulfment by both amoebae and macrophages^{2, 111, 112}.

5.1. A *Dictyostelium* Model

The eukaryote *Dictyostelium discoideum* is known as a social amoeba or cellular slime mold. In the unicellular phase of its life cycle, it is a free-living amoeba that can feed on a variety of bacteria. *P. aeruginosa* and *Bacillus anthracis* are particularly poor food sources¹⁰¹. In *Dictyostelium* liquid growth medium, amoebae consumed *P. aeruginosa* PA14 cells 100 times more slowly than cells of *Klebsiella aerogenes*, a bacterial food source for amoebae in the laboratory⁹⁶. In a PA14 lawn at room temperature, most amoebae were killed within 24 hr.

P. aeruginosa quorum sensing contributed to its virulence towards amoebae. Amoebae could survive in PA14 lawns generated on agar with reduced nutrient concentration⁹⁶, the lower bacterial density resulting in reduced expression of quorum-sensing controlled genes. Furthermore, amoebae survived in a *lasR* mutant lawn at room temperature, making visible plaques after 5 days as they fed. This virulence defect was not explained by the reduced pyocyanin production in the *lasR* mutant, since purified pyocyanin (in an amount equivalent to that generated by PA14) did not kill amoebae. The role of the *rhl* quorum sensing system in virulence towards amoebae was revealed using *P. aeruginosa* PAO1²⁰. The virulence defect of *rhl* regulatory mutants was partly explained by the consequent decrease in rhamnolipid production. Purified rhamnolipid lysed amoebae in minutes, as did spent culture supernatant of the parental PAO1 strain. Inactivation of the rhamnolipid biosynthesis gene *rhlA* eliminated the lytic activity in these supernatants.

Two mutants of *P. aeruginosa* PA103, originally identified as strains impaired in killing epithelial cells⁶¹, were also tested in the amoeba assay: A *pscJ* mutant and a strain lacking the Type III effector ExoU⁹⁶. Both strains were less virulent, with amoebae surviving and making visible plaques in lawns of each mutant. The *pscJ* gene encodes a structural component of the Type III secretion apparatus. ExoU is a lipase cytotoxin¹⁰³ that is produced only in some *P. aeruginosa* strains and not in PAO1, and whose role in virulence was also detected in the *G. mellonella* model⁸⁵.

6. FUNGI

Pseudomonas aeruginosa-secreted products such as cyanide and phenazines have antifungal activity suggesting an adversarial relationship between the two organisms⁴⁸. Nevertheless, *P. aeruginosa* stands to benefit from a close association with fungi. The bacterium is well endowed with pathways for catabolism of aromatic compounds released after the initial degradation of plant material by fungi⁵⁸. *P. aeruginosa* may contribute to the microbial ecology of the human body through its interactions with fungi such as *Candida albicans*, an opportunistic pathogen⁵⁴.

6.1. A *C. albicans* Model

Candida albicans strain SC5314 became a food source when it was mixed with a suspension of *P. aeruginosa* PA14 cells diluted in PA14 spent culture supernatant and incubated at 37°C⁵⁴. Under these conditions, *P. aeruginosa* colonized and killed *C. albicans* filaments, while leaving the yeast form of the fungus largely untouched. Filamentous growth of *C. albicans* is thought to contribute to its role as an opportunistic pathogen. As in its interactions with human epithelial cells and plant cells, PA14 initially attached by one cell pole to fungal cells, and this was observed with strain PAO1 as well. PA14 mutants attenuated in virulence in other model systems were tested for their ability to kill a constitutively filamentous *C. albicans* mutant⁵⁴. Most of these strains showed attenuated killing based on fungal CFU measured 42 hr after addition of bacteria. Such strains included *gacA*, *lasR*, *rhlR*, and *phnAB* mutants, each impaired in a global regulatory system. A *plcS* mutant was also impaired in killing *C. albicans*, but not a mutant lacking exotoxin A.

7. VIRULENCE DETERMINANTS

P. aeruginosa virulence determinants identified using non-vertebrate model organisms included both regulatory systems as well as specific toxins.

The impaired virulence of *P. aeruginosa* mutants was explained by the loss of a single toxic activity in only one case: Paralytic killing of *C. elegans* by cyanide poisoning. In the other assays, there was evidence for multiple virulence factors. A mutant lacking exotoxin A, for instance, was less virulent in several of the assays but never avirulent (Table 1), and the action of either of the Type III effectors ExoT and ExoU could mask the contribution of the other⁸⁵. In other cases, such as the contribution of rhamnolipid or ExoU to amoeba killing, the relevant virulence factors depended on the *P. aeruginosa* strain tested^{20, 96}. However, even using derivatives of a single strain (PA14) in multiple model systems, surprising patterns were revealed: The Type III secretion system was involved in virulence towards caterpillars but not in virulence towards *Arabidopsis* or *C. elegans*⁸⁵. In contrast, the quorum sensing system and the GacA-GacS two-component regulatory system overlap in their regulatory targets¹⁰² and played a role in virulence towards all of the organisms assayed (Table 1). The contribution of regulation by PQS, an extracellular signal that interacts with quorum sensing, was revealed by mutants blocked in PQS biosynthesis^{15, 26, 42} due to a mutation in *mvfR*, *phnAB*, or *pqsB* (Table 1).

7.1. Ecological Role

Before its intentional use in model systems of virulence, *P. aeruginosa* was identified as the cause of disease not only in humans but also in crop plants³⁰ and grasshoppers¹². Its ability to cause disease in humans, and its broad host range in general, may reflect the accumulation over evolutionary time of successful adaptations to the challenges of interacting with progressively more complicated eukaryotic organisms including plants, insects, nematodes, protozoa, and fungi. *P. aeruginosa* virulence determinants have an ecological role, enabling the bacterium to withstand predation, overcome competition, and exploit opportunities^{33, 54}. The environments where *P. aeruginosa* is encountered may represent dispersal from an as yet undiscovered specific niche, perhaps one in which it has evolved to interact with a plant or a fungus. Alternatively, it may be an opportunist by nature, as *P. putida* is a biodegradative opportunist that tends to overgrow other bacteria in enrichments based on growth with exotic or toxic compounds¹²⁰.

Perhaps the most ancient selective pressure acting on bacteria arose from interactions amongst themselves. Without competition between bacteria, *P. aeruginosa* might much more often play the role of opportunistic pathogen. *P. aeruginosa* became a major cause of human burn wound sepsis only after the development of effective anti-staphylococcal therapy¹⁰, and in a cystic fibrosis mouse model of chronic lung infection, indigenous *Enterobacter* spp. interfered with initial colonization by *P. aeruginosa*¹⁸. *P. aeruginosa* extracellular factors that have antibiotic activity include phenazines, pyocins, and a variety

of quinolones closely related to PQS^{13, 50, 72, 75, 84, 105, 121, 134}. Following a line of investigation begun by Pasteur, concentrated sterile *P. aeruginosa* spent culture supernatant was shown to prevent anthrax in rabbits⁵⁰. This preparation (called pyocyanase) was used clinically as an antibiotic long before the discovery of penicillin. *P. aeruginosa* pyocins were first studied by Jacob in 1954, and the killing activity of R-type pyocins has been used to study cell surface lipooligosaccharides of species of *Haemophilus* and *Neisseria*⁸⁴. It is likely that many new aspects of the biology of *P. aeruginosa* will be revealed by studying its interactions with other bacteria.

7.2. Physiological Role

The economy of bacterial physiology and the ad hoc nature of evolutionary change conspire in endowing bacterial products and regulatory systems with more than one function. Bacterial opportunists may be the culmination of this process. The phenazine pigment pyocyanin is a redox agent that not only is toxic to other bacteria and eukaryotic cells, but also can enhance respiration in *P. aeruginosa*³⁹ and protect against damage by sunlight³⁵. The LasB protease, a secreted virulence factor under control of the *las* and *rhl* quorum sensing systems, is involved in tissue degradation. It also functions intracellularly in *P. aeruginosa* to activate the nucleotide diphosphate kinase Ndk for GTP generation, and therefore plays a role in various stress responses^{16, 60, 65}. The *rhl* quorum sensing system regulates the expression of extracellular toxins as well as intracellular factors that prevent a toxic accumulation of nitric oxide in *P. aeruginosa* during anaerobic growth¹³². Cyanide gas, an effective poison, may have a regulatory role in *P. aeruginosa*, given that an *hcnC* mutant overproduced pyocyanin⁴¹. A precedent for such a role is the confinement sensing system based on the volatile fatty acid derivative 3-hydroxypalmitate methyl ester that regulates virulence in the bacterium *Ralstonia solanacearum*, a pathogen of plants¹⁰⁴.

Individual *P. aeruginosa* virulence determinants played a role in multiple model organisms (Table 1). For virulence determinants such as exotoxin A and substrates of the Type III secretion system, this likely reflects a common mechanism of action in the different models. For others, this may reflect at least in part their physiological role in *P. aeruginosa*. Consistent with a fundamental physiological role for quorum sensing, mutants defective in this regulatory system were genetically unstable during growth in the laboratory. Mutation of the *las* system selected for *vfr* mutants, and mutation of the *rhl* system selected for *algR* mutants^{8, 9}. The basis for this selection is unknown, although the fact that *algR* mutants have enhanced resistance to oxidative stress is suggestive⁷⁶. Vfr is a homolog of the *E. coli* cyclic AMP receptor protein CRP, and acts at the top of the quorum sensing regulatory hierarchy⁸.

The genetic instability of quorum sensing mutants was discovered because of the colony morphology associated with loss of twitching motility due to mutation of *vfr*^{8, 9}. The genetic instability of other mutants identified in virulence assays was also readily visible. A *cbrA* mutant was less virulent in the *C. elegans* paralytic killing and fast killing assays^{41, 116}, and the PAO1-derived strain was noted to form lawns with abundant papillae, indicating impaired viability of mutant cells and selection for faster growing derivatives⁴¹. The *cbrA* gene (PA4725 in strain PAO1) encodes a central catabolic regulator⁸⁷. Physiological imbalances, even those without a detectable effect on cell growth or viability, may complicate the interpretation of results from assays with protracted incubation of *P. aeruginosa* mutants. Indeed, for mutations inactivating virulence determinants with a physiological role, indirect and direct effects may be difficult to distinguish.

7.3. PAO1 Strain Variation

In addition to the genetic instability of certain *P. aeruginosa* mutants, laboratory isolates of the reference strain PAO1 themselves vary. The sequenced isolate of PAO1 has a spontaneous 8-bp tandem duplication that inactivates *mexT*^{67, 80}. MexT is a transcriptional activator of genes encoding a multidrug efflux pump. This pump confers resistance to chloramphenicol and quinolones, and its substrates appear to include PQS⁶⁷. PAO1 isolates in which *mexT* is inactivated secrete more quorum sensing controlled virulence factors consistent with intracellular accumulation of PQS. PAO1 isolates with a functional *mexT* (designated NfxC) are less virulent²⁰. The identification of particular virulence determinants in PAO1 may therefore depend on the allele of *mexT*. PAO1 from the Iglewski laboratory has a functional *mexT*⁶⁷ and was used in the nematode paralytic killing and fly wounding assays^{27, 41}. The sequenced PAO1 isolate also lacks twitching motility, possibly because of a spontaneous mutation in *pilC*, and carries an inversion of more than a quarter of the genome relative to other PAO1 isolates¹¹⁵.

8. MODEL SYSTEM DEVELOPMENT

The model systems of *P. aeruginosa* virulence, although only recently developed, have already led to the identification of known virulence determinants (that can now be studied in a new context) as well as genes of unknown function. To reveal additional *P. aeruginosa* virulence determinants, including those that either are redundant or confer a subtle affect, several strategies can

be used. Techniques based on in vivo expression technology (IVET) will be particularly valuable^{127, 128}. Virulence towards one of the model organisms can be partially reconstructed²² using a relatively avirulent bacterium (such as *E. coli* or *P. putida*) expressing cloned DNA from *P. aeruginosa*. Transposon insertion mutants can be screened using as a parental strain either a *P. aeruginosa* mutant already partially impaired in virulence or a hypervirulent strain such as the *qscR* mutant in the fruitfly feeding assay¹⁷. All of the *P. aeruginosa* mutants identified using the non-vertebrate model organisms can subsequently be tested in various mouse models of infection^{18, 77, 131}.

The genetic tools available for the model organisms will facilitate the isolation of mutants more resistant to *P. aeruginosa*, thereby revealing antimicrobial strategies likely to have been refined over millions of years of evolution¹¹⁶. Drugs can be screened for those that suppress *P. aeruginosa* virulence in the model systems or that enhance a beneficial host response^{36, 59}. A mixture of bacterial pathogens⁴⁶, perhaps more relevant to clinical situations, could be used in such assays. DNA microarray analysis, made possible by the genome sequences of the model organisms, can reveal the host response to interactions with *P. aeruginosa*⁵⁶.

9. CONCLUSIONS

The *P. aeruginosa* genome is particularly rich in genes encoding transporters and regulatory proteins, consistent with a physiology finely tuned for rapid adaptation to diverse environments¹¹⁵. *P. aeruginosa* virulence in diverse model systems reflects this adaptability in action. The quorum sensing system, PQS, and the GacA-GacS two-component system form part of a complex regulatory network^{8, 53, 91, 92, 102, 106, 124, 126, 130} that underlies virulence as well as physiological adaptability. Elements of this regulatory network are therefore attractive targets for new antibiotics^{6, 49, 52, 90}, both in *P. aeruginosa* and in other bacterial pathogens of humans⁹⁰. Such antibiotics, while not killing the bacteria, could be used in conjunction with existing drugs and in the early stages of chronic infections might be particularly useful to hamper the bacterial adaptability that underlies persistence.

P. aeruginosa was given its species name because of the blue-green color of its pigments. This color is also the basis for one of its earliest described phenotypes: The chameleon phenomenon, in which cultures of *P. aeruginosa*, when disturbed, change their color as pyocyanin is reversibly oxidized¹²¹. The challenge of revealing the many faces of *P. aeruginosa* was thus foreshadowed from the outset. Pursuing this challenge remains the best strategy to reveal the vulnerabilities associated with life as a bacterial opportunist.

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PSEUDOMONAS AERUGINOSA **INTERACTIONS WITH HOST CELLS**

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1. INTRODUCTION

The opportunistic nature of many different *Pseudomonas aeruginosa* infections leaves patients with compromised immune systems highly susceptible to infection. Common forms of immune suppression leading to *P. aeruginosa* infection include burn and trauma wounds, diseases such as AIDS, or chemotherapeutic treatments that affect leukocyte numbers and functions. Cystic fibrosis (CF) patients develop chronic life-threatening lung infections with *P. aeruginosa* and can be considered to have defective innate and acquired immunity to this organism. Although rare, people who use extended-wear soft contact lenses are at an increased risk for acute ulcerative keratitis.

Bacterial and host factors implicated in pathogenesis of *P. aeruginosa* infection have been recently reviewed¹⁰⁷. Some of the molecular aspects of the interactions between *P. aeruginosa* and host cells are conserved in these various infections and others appear specific. Importantly, but also problematically for

the design of prophylactic and therapeutic strategies, *P. aeruginosa* possesses a broad array of virulence factors, many of which have redundant functions and thus their loss does not result in much attenuation of disease. Under appreciated, but also critically important, is the variation in genome structure^{100, 156, 170} and virulence factor elaboration among clinical and environmental isolates of *P. aeruginosa*. For example, while it is clear that production of the effector known as exotoxin U (ExoU), secreted by the type III secretion system increases virulence in animal studies of infection^{2, 94}, only a minority of *P. aeruginosa* isolates produce this toxin¹³⁶ and there appears to be little difference in the pathology due to ExoU⁺ strains compared with those that do not make this toxin. Similarly, while possessing the ability to transmigrate through epithelial tissues and cause systemic infection leads to a severe disease state, significant pathology emanates from *P. aeruginosa* strains that remain localized in tissues such as the lung⁷³. Overall it is very challenging to define a core set of bacterial virulence factors and host susceptibility states important in *P. aeruginosa* infection because providing such a definition is affected by the genetic and phenotypic versatility of *P. aeruginosa* isolates and the ability of many different host immune effectors, including those of the innate immune system and the humoral and cell-mediated factors of the acquired immune system, to modulate disease. Nonetheless some major insights have been garnered from recent molecular and cellular studies that reveal the way in which *P. aeruginosa* interacts with host cells, providing a framework for more detailed analysis of the signaling pathways and transcriptional responses of both host and pathogen that should shed significant light on the pathogenic process related to *P. aeruginosa* infections.

2. SOME GENERAL CONSIDERATIONS

2.1. Implications of Binding and Internalization of Bacteria by Host Epithelial Cells

One often reads in the literature on microbial pathogenesis that a pathogen must adhere to host cells in order to initiate the pathogenic process. However, a close scrutiny of studies indicates that in fact this statement oversimplifies a complex process wherein binding and uptake of bacterial pathogens often leads to effective host immune resistance while elaboration of virulence factors, phase variations, and other microbial responses can circumvent the production of resistance factors and responses, and promote colonization, infection, and potentially disease. An additional problem in trying to study bacterial–host cell interactions in a defined and controlled way is that most pathogens elaborate a range of adhesins that can be used to bind to host cells, as well as enzymatic and other factors that modify host cells to facilitate

pathogen interactions. Thus interrupting or inhibiting one or even a few microbial factors may have little effect on interactions with cells. Perhaps more importantly, the binding of bacterial pathogens to host cells initiates a dynamic host response, often leading to effective host innate immune responses that rid the body of the pathogen, and only rarely leading to colonization, infection, and ultimately disease. For example, it is not well appreciated, but nonetheless clear from studies of numerous, principally extracellular, bacterial pathogens, that virulent strains avoid binding and internalization by host epithelial cells lining mucosal surfaces. Thus, when production of a well-defined virulence factor such as the capsule is interdicted in *Haemophilus influenzae* type B¹⁵⁷, *Klebsiella pneumoniae*²⁶, group A *Streptococcus*¹⁴⁸, and *Neisseria meningitidis*^{33, 166}, for example, there is increased epithelial cell binding and uptake of the pathogen. Since these unencapsulated forms are poorly virulent it appears that the capsule provides protection from epithelial cell, as well as phagocytic cell, binding and ingestion; avoidance of uptake by both types of cells is important for maintenance of full virulence. Similarly, it has been shown in both *Escherichia coli* bladder infections¹¹³ and *P. aeruginosa* lung infections⁵⁹ that binding and internalization of bacteria by epithelial cells is part of the clearance process, and when bacterial levels are achieved that overwhelm this innate response, then infection and disease ensues. Therefore, it is important to keep in mind when studying bacteria–host cell interactions that the host uses this dynamic interaction to activate innate immune effectors to eliminate the pathogen, and can also use this interaction to initiate acquired immune responses. Circumventing or overwhelming effective host innate immunity is needed for an organism's pathogenic potential to become realized.

2.2. Does *P. aeruginosa* Actually Bind to Host Epithelial Cells during Infection?

Surprisingly there are only limited data to indicate that *P. aeruginosa* binds to host epithelial cells as part of the pathogenic process in vivo. Importantly, in vivo binding to epithelial cells is more strongly associated with increased bacterial clearance and enhanced host resistance to infection, at least in mice¹⁵⁰. If anything, the small amount of data available from animal and human studies suggests at best transient binding of *P. aeruginosa* to host cells and subsequent immune clearance associated with this binding^{121, 122, 150}. Almost all of the information about host cell binding interactions comes from in vitro studies with cell and organ cultures. However, given the clear role of type III secretion factors in virulence^{3, 5, 23, 28, 136, 164} whose delivery is dependent on bacterial binding to host cells and injection of effector toxins, there must be some critical *P. aeruginosa*–host cell interactions that are important for the manifestation of the effects of these toxic factors.

In vivo studies of tracheal and corneal tissues obtained from experimentally infected animals do show *P. aeruginosa* in and on epithelial cells of wild-type animals^{150, 175}, but these studies also suggest this is a transient process leading to bacterial clearance. What is more consistently found in human and animal studies, however, is that *P. aeruginosa* is found in highest numbers trapped within the mucus layer that lines the epithelium and particularly in CF, but less so in wild-type situations, rarely attached to host cells. It has been known for over a decade that microscopic observations of *P. aeruginosa* in the lungs obtained from CF patients at autopsy or transplant show few, if any, *P. aeruginosa* adhering to epithelial cells with most of the bacteria trapped within mucus plugs in the airway lumen^{10, 80}. More recent studies have completely confirmed this finding^{13, 173}. In a murine model of *P. aeruginosa* gastrointestinal colonization, following depletion of normal aerobic Gram-negative flora with antibiotics, the bacteria were found principally within the mucus layer¹²³. A comparison of *P. aeruginosa* adherence to the tracheal epithelium in wild-type and transgenic CF mice only noted bacteria associated with the epithelium in the wild-type animals, not the CF animals¹⁵⁰. In organ cultures of human adenoids *P. aeruginosa* was seen rarely associated with the epithelium but rather with areas of denuded epithelium and in the mucus¹⁶³. Overall, while there is some evidence from murine studies that *P. aeruginosa* in a highly resistant wild-type mouse has a transient interaction with the lung epithelium, almost all of the studies involving transgenic CF mice or lung tissues from humans with CF fail to find any association of *P. aeruginosa* with lung epithelial cells. Thus, it seems likely that if this does occur it is transient and is part of the wild-type host response involved in rapid elimination of the microbe from the respiratory tract.

Similarly, in studies of the pathogenesis of *P. aeruginosa* eye infections many accounts show the adherence of the organism to epithelial cells and organ cultures performed in vitro^{52, 53, 65, 72, 138, 153} but few studies have actually looked at eyes removed from infected animals. Fleiszig *et al.*⁴⁹ and Zaidi *et al.*¹⁷⁵ did show the intracellular presence of *P. aeruginosa* in corneal epithelial cells of mice with scratch-injured and infected eyes, suggesting that there had to be an initial bacterial binding to a receptor. Interestingly, in the latter study, the inhibition of the binding and entry of the bacteria into the corneal cells was associated with less inflammation and less disease pathology, indicating that in the murine scratched injured eye model epithelial binding does serve as a component of virulence. But this is a situation that may be unique to the eye, and perhaps even to this model of keratitis. In this model, the bacteria travel down the scratch injury made in the cornea to the basal epithelial layer and enter corneal cells buried at the bottom of the epithelium that is made up of about 5–6 cells lying on top of each other⁴⁹. When entrapped within the buried cells, the bacteria are resistant to host immune effectors, particularly

phagocytes, and thus serve as a nidus for continued infection, virulence factor elaboration, and inflammation, all contributing to pathology and disease.

2.3. Measuring Adherence and Adhesins

In spite of the *in vivo* observations, many investigators have looked at *P. aeruginosa* adherence to cells grown *in vitro* and reported significant effects that may be involved in either pathogenesis or host immunity. Bacteria can bind to host cells using a variety of microbial adhesins and a comparable variety of host receptors. However, measurements of this interaction, while very common in the field of pathogenesis, can be fraught with severe difficulty; many problematic or confounding aspects of these adherence assays are not usually considered. For example, as adherence is a dynamic process governed by the law of mass action, one rarely sees conditions tested or association and dissociation constants measured that give a quantitative assessment of the strength of the interaction. An organism with a low association and dissociation rate might show low adherence in a short assay as the binding rate is low, but because the dissociation rate is also low may accumulate in high numbers if enough time passes during the assay. Similarly an organism with a high dissociation rate might appear to bind more in a short-term assay as opposed to a long-term one. Many adherence assays are performed with a standard "3 washes" to remove non-adherent bacteria but little is usually done to quantify the bacterial levels in the wash to determine that what is coming off represents a small, biologically negligible and diminishing amount of organisms. For example, Schroeder *et al.*¹⁵¹ found it took 6–8 washes of epithelial cells to which *P. aeruginosa* was added before the level of bacteria being removed had stabilized at <0.01% of what was subsequently measured to be the adherent population. Epithelial cells in culture may trap bacteria underneath the cells without any actual contact, making the microbes resistant to removal by washing but being counted as part of the adherent population upon detachment of the epithelial cells from their substrate or following lysis of the epithelial cells.

Whether cells are grown as regular tissue-culture monolayers or on thick or thin gel supports¹²⁶ or as polarized monolayers on transwells greatly affects *P. aeruginosa* binding^{46, 95} and addition of growth factors or other media components can alter binding of bacteria to cells⁴⁷. One problem with use of polarized cells grown on transwells⁹⁵ or *P. aeruginosa* grown on thick collagen gels¹²⁶ is that the tight junction formation may not be representative of what occurs with tissues *in vivo*. Lee *et al.*⁹⁵ achieved trans epithelial resistances (TER) of >1000 ohms/cm² when growing Calu-3 cells on transwells and found little interaction with *P. aeruginosa*. When the TER was reduced by treatment with EDTA to lower levels then increased interactions were

observed. Barker *et al.*¹¹ reported the TER of the lung epithelium is around 360 ohms/cm², indicating that studies with polarized cells with TER much higher than this are not likely representative of the *in vivo* situation. More to the point, numerous *in vivo* studies with murine and monkey tissues^{121, 122, 149, 150, 175} show *P. aeruginosa* binding, internalization and epithelial-cell activation, indicating that the polarized epithelium on intact tissues can interact with *P. aeruginosa* in a rapid and robust fashion (Figure 1). It has also been noted that *P. aeruginosa* tends to enter not only mammalian epithelial cells in culture, but even plants¹²⁷ and fungi⁷⁶ in a polarized fashion with the pole opposite that of the flagella entering into the cell first.

Additionally, the presence of other cells in cultures of polarized epithelial cells, particularly those that can disrupt tight junctions, even briefly, may be important. Investigations using polarized cells and *P. aeruginosa* in binding and internalization usually report this phenomena when the bacterium gains access to the basolateral membrane, but not when there is only access to the

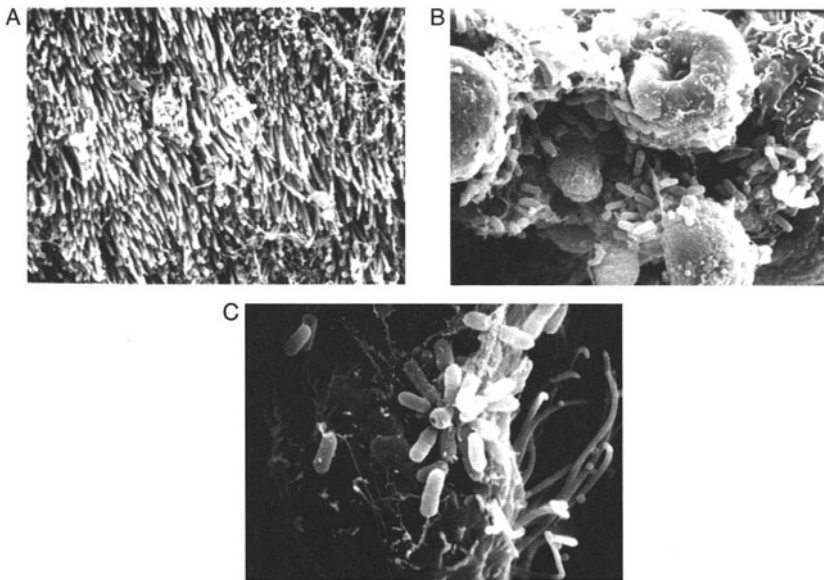


Figure 1. *P. aeruginosa* entering monkey tracheal cells. Uninfected monkey trachea (X 6250, Panel A). Monkey tracheas 6 hr post infection with *P. aeruginosa* strain PAO1 (X 6250, Panel B; and X 12,500, Panel C). Bacteria can be seen attached and entering cells in a polarized fashion. The cells have either started to round up (Panel B) or desquamate (panel C) from the epithelial surface, producing areas of epithelial destruction. Many of the cells to which bacteria bind do not have obvious cilia, although some do (Panel C). It is not known if this is due to preferential binding to non-ciliated cells or rapid loss of cilia from cells attacked by *P. aeruginosa*.

apical membrane^{46, 95}. But on an epithelium in vivo, there is a dynamic cellular response and trafficking for organisms like *P. aeruginosa* that induce a robust mast cell^{14, 103, 104} and polymorphonuclear leukocyte (PMN) response to infection. Mast cells respond rapidly to *P. aeruginosa*, producing IL-1 which promotes the transmigration of PMN through the endothelium to the basal side of the epithelium from where they can further move to the apical side of the cells, a process that may be sufficient to allow bacterial access to the basolateral membranes. Sansonetti and colleagues¹²⁰ found that *Shigella* will not enter polarized enterocyte monolayers unless PMNs were added to the basal side of the culture. Almost all studies of *P. aeruginosa* using polarized epithelial cell cultures fail to include PMN in the system, and it is likely that even a brief disruption in tight junctions that occurs as the PMN transmigrate through the epithelium is sufficient to allow *P. aeruginosa* access to the basolateral membranes. Overall, what is and is not determined to be a significant bacterial or host component to the microbial–host cell interaction can be highly variable and dependent on numerous factors when using cell cultures, and confirmation with appropriate in vivo studies using both laboratory strains and clinical isolates of pathogenic bacteria needs to be vigorously pursued.

2.4. Measuring Internalization

Bacterial internalization, also commonly referred to as invasion, is a mechanism for both host elimination of pathogens as well as one involved in causing disease and avoiding the host immune response. The standard technique for measuring internalization was developed by Falkow and colleagues⁷⁹ where they added the antibiotic, gentamicin, to tissue culture cells after infection with *Yersinia pseudotuberculosis* and killed those bacteria residing outside the eukaryotic cell. Subsequent lysis of the eukaryotic cells resulted in release of the bacteria that could then be enumerated. While an extraordinary amount of useful data has been generated using this technique, there are some considerations when applied to *P. aeruginosa* in particular. For example, because of the inherent antibiotic resistance of *P. aeruginosa*, high levels of gentamicin are required to kill this organism and in the context of a tissue culture system where bacterial levels may reach 10⁹ cfu/well the levels of bactericidal antibiotic may be even higher. Importantly, in the early papers reporting *P. aeruginosa* invasion of eukaryotic cells it was emphasized that control wells were needed to ensure that a proper amount of antibiotic had been added^{49, 50}. In these wells, cells were lysed after the internalization step to release all the bacteria, then antibiotic added and the reagents incubated together. However, it was reported that mere plating of this solution to insure high level killing of *P. aeruginosa* was not sufficient; and, in fact, the solutions in these control wells had to be centrifuged to pellet the bacteria and leave the antibiotic behind

in the supernatant. Resuspension of the pellet (usually too small to be seen) in antibiotic-free media and plating revealed the efficacy of the treatment. Plating the intact solutions without centrifuging the bacteria out allowed the antibiotic to continue its effect, providing a false sense that all bacteria were dead when in fact they were not. Another means to verify the antibiotic efficacy is to look at hanging drop suspensions of the organisms in a microscope; as long as the *P. aeruginosa* under study is motile it will be seen swimming if still alive, but moving only by Brownian motion if dead.

In addition, care must be taken to assure that antibiotics have no effect on the eukaryotic cells in culture. This can be assessed by performing replicate experiments with different antibiotics to which the organism is susceptible and using only those that do not enter cells (i.e., an aminoglycoside or carbenicillin). Additional problems exist when polarized cells are used to determine the role and localization of cellular receptors. Penetration of antibiotics through or between these cells may not provide discrimination of apical or basolateral entry of bacteria. In general, all these *in vitro* assays should be validated with additional experiments such as using GFP-labeled bacteria or staining the bacteria, before and after cell lysis, followed by confocal microscopy to determine whether bacteria are actually internalized. A simple method we have found to visualize *P. aeruginosa* by confocal microscopy is to incubate the organism for about 30 min prior to an experiment with a fluorescent dye such as fluorescein, Texas Red, or 4,6-Diamidino-2-phenylindole dihydrochloride (DAPI). These dyes are readily taken up and do not affect the organism's properties, but in order to separate internalized bacteria from the free dye we found the bacteria had to be washed about 8 times.

3. SITES OF INTERACTION

The ubiquitous occurrence of *P. aeruginosa* in the environment makes virtually any portal of entry into the human body a potential site of attachment. In almost all instances, *P. aeruginosa* is a common infectious agent associated with both epithelial and mucosal surfaces. *P. aeruginosa* is most notorious for its ability to cause lung infections, indicating a clear predilection for this tissue. It is obviously the overwhelming pathogen in lung disease in CF, and is usually the first or second most common cause of nosocomial pneumonia^{134, 135}. Colonization of the gastrointestinal (GI) tract occurs as a consequence of antibiotic administration and often chemotherapy in immunocompromised patients^{92, 93} and there is a great deal of controversy as to whether selective decontamination of the gastrointestinal tract with topical antibiotics affects the development of nosocomial pneumonia. The damaged corneal epithelium is

highly susceptible to *P. aeruginosa* infection, particularly after trauma or associated with extended wear contact lenses that are not removed at night^{45, 169}. Otitis externa or swimmers ear is frequently due to *P. aeruginosa* infection of the auditory canal¹⁶⁷. *P. aeruginosa* is the third or fourth most common cause of urinary tract infections in catheterized patients^{134, 139}. Finally, *P. aeruginosa* is also a significant cause of catheter-related infections, utilizing this route of entry from the environment to the interior of the host to establish infection^{36, 134, 135}. What this means is that *P. aeruginosa* is highly versatile in its ability to interact with many different host tissues and a significant proportion of bacterial infectious diseases results from this versatility.

4. ATTACHMENT

4.1. Adherence to Host Tissues

In the lung, it appears that *P. aeruginosa* may only attach transiently to the epithelial cells, and most of the microbial cells are found within the mucus layer lying just above the epithelium (Figure 2). The composition of mucus

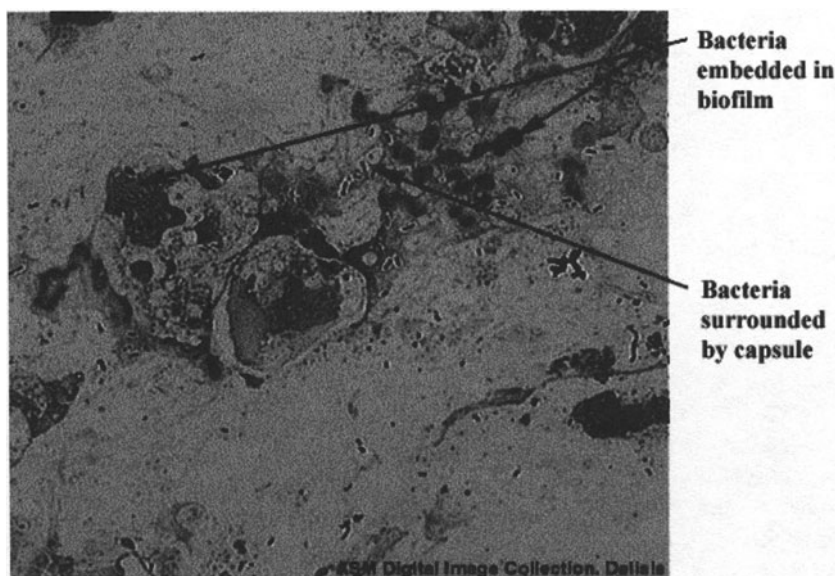


Figure 2. *P. aeruginosa* growing as a biofilm in the sputum of a CF patient. Small clusters of bacteria are visible embedded in an extracellular matrix that could be called a biofilm. Also seen are clear halos surrounding the organisms, indicative of a capsular-like material surrounding the bacteria.

may impact the interaction with bacteria. In the CF lung, the mucus has increased sulfation¹⁰⁵ and recent studies indicate that sialyl-Lewis X and sulfo-sialyl Lewis X blood group antigens are major sites of bacterial attachment^{145, 146}. The primary *P. aeruginosa* adhesin for respiratory mucins is the flagella cap protein, FlhD^{6, 7}. In other studies, the flagellin itself was shown to be the adhesin for binding to Muc1 mucin on epithelial cells¹⁰¹. In these studies, strain PAK and flagella mutants were tested for their adherence to either CHO cells or CHO cells transfected with Muc1 cDNA. The wild-type strain showed increased binding to the CHO–Muc1 cells, whereas a mutant lacking the flagellin did not show this increase. Binding by flagella was inhibited by flagella antisera and purified flagellin. This appears to be independent of the flagella type as both a-type (PAK) and b-type (PAO1) showed increased adherence to CHO–Muc1 cells. However, many strains of *P. aeruginosa* isolated from long-term, chronically infected CF patients do not produce flagella¹⁰⁸ suggesting that mucin binding is an early step in colonization of the CF lung and longer term maintenance of the organism in this milieu may involve other factors.

It has also been reported that *P. aeruginosa* infection can influence mucin expression. Kohri *et al.* have shown that culture supernatants induce mucin Muc5AC expression via the epidermal growth factor receptor⁸⁹. Similarly Bausbaum and colleagues have found *P. aeruginosa* induction of mucin secretion from cells cultured in vitro^{98, 99} and this occurs via activation of NF- κ B and a Src-dependent mitogen-activated protein (MAP) kinase pathway. In a wild-type setting, mucin secretion, particularly if it is CF transmembrane conductance regulator (CFTR)-dependent, is probably part of the normal host response to remove the bacteria from the lung⁶⁰. In the setting of CF, however, increased mucin secretion, in a CFTR-independent fashion, and formation of dehydrated mucus can become a setting for adherence of *P. aeruginosa* and poor clearance of the mucus plugs then serves as the “home” for the developing biofilm and eventually for emergence of the mucoid phenotype. Thus the organism seems to take advantage of the host’s innate response to promote its ability to colonize the lung.

The study of specific receptors in the lung has been virtually completely confined to cell cultures. The relevance of this to actual lung infection is questionable, particularly in the case of CF where consistently no *P. aeruginosa* binding to lung epithelial cells is observed in lung samples from patients. What binding is observed is usually to basement membrane or the damaged epithelium^{10, 13, 31, 32, 80, 126}. While some proponents of a role of *P. aeruginosa* binding to epithelial cells in the pathogenesis of CF lung disease have explained the observations in patients as reflecting what occurs late in disease and not early on during the establishment of infection, this explanation is still completely speculative as samples from nosocomial or CF lung disease early in the course of infection have not been evaluated. In contrast to the

human situation, studies of acute infections in wild-type and transgenic CF mice showed clear adherence of *P. aeruginosa* to the tracheal epithelium 4 hr after intranasal application of bacteria in wild-type mice but no such adherence could be found in transgenic CF mice¹⁵⁰. Certainly, while some animal studies suggest that the loss of *P. aeruginosa* adhesins such as flagella and pili decrease pathogenesis of lung infection^{43, 160, 161}, these studies can never attribute the loss of virulence directly to loss of binding to host cell receptors. Many other changes to the organisms' virulence accompany loss of these adhesins, notably motility and ability to form biofilms¹¹⁴. Thus, essentially no animal or human study has shown that it is essential for *P. aeruginosa* to adhere to epithelial cells in order to establish infection. Indeed, it appears that binding of *P. aeruginosa* to receptors on lung epithelial cells is more likely to activate host defenses and promote bacterial clearance¹⁰⁷.

Nonetheless, many investigators have suggested that asialo GM1 is a receptor for *P. aeruginosa* and its expression is increased on CF cells^{18, 29, 91, 128, 142, 143}. Both pili and flagella have been shown to bind to asialo GM1; however as the expression of these bacterial ligands is lost by at least some of the infecting *P. aeruginosa* cells during chronic infection, these receptors, if relevant, are likely only critical during the initial stages of colonization and may be used by only a subset of the cells in a population. In spite of numerous different investigators finding a role for asialo GM1 in binding of *P. aeruginosa*, and increased expression on CF cells, there are serious problems with these data that question whether it is a consistent phenomenon. For example, in the early studies identifying asialo GM1 as a cellular receptor for *P. aeruginosa* on CF epithelial cells, differences in binding between wild-type and CF cells were only observed when very high concentrations of bacteria were present: $>10^8$ cfu/culture well^{142, 143}. While this level of bacteria can be achieved in sputa of chronically infected CF patients it is exceedingly unlikely to represent an achievable concentration early on in infection when it has been proposed that binding of *P. aeruginosa* to asialo GM1 is important. Furthermore, while the exact multiplicity of infection used in these binding experiments was not stated, most tissue culture wells hold only 10^5 – 10^6 cells, so the ratio of bacteria to cells was likely in the range of 100:1–1000:1, another indication that this level of infection is likely not relevant in vivo. Also, when nasal epithelial cells from CF patients with a variety of different mutations in *CFTR* were studied for binding of *P. aeruginosa*, enhanced binding was only associated with cells from patients homozygous for the $\Delta F508$ *CFTR* allele¹⁷⁶. Since patients with the other genotypes, many of which were compound heterozygotes with at least one $\Delta F508$ allele, get the same disease it is not clear why enhanced expression of asialo GM1 on cells from only 50% of CF patients homozygous for the $\Delta F508$ *CFTR* allele would be a component of the pathogenic process.

Another problem related to whether asialo GM1 is a receptor for *P. aeruginosa* is that most of the studies have used laboratory strains of this organism such as PAO1 and PAK, while Schroeder *et al.*¹⁵¹ found no increased binding of clinical isolates of *P. aeruginosa* to asialo GM1. Much more problematic for these studies is that they have almost all used a commercially available antibody to asialo GM1 to show inhibition of adherence of *P. aeruginosa* to this receptor. But as was recently reported¹⁵¹, this antibody is raised to asialo GM1 isolated from bovine tissues, emulsified in complete Freund's adjuvant (CFA) and methylated BSA and used to inject rabbits. The antibody has high titers to both BSA and numerous bacterial antigens due to cross reactivity with the *Mycobacterium tuberculosis* present in the CFA and in few studies was the specificity for asialo GM1 shown by using the purified antigen to neutralize the biologic effects of the inhibitory antibody. Further recent reports indicating that ligation of asialo GM1 activates host cell responses^{97, 132} are similarly confounded by lack of evidence to confirm that it is the antibody specific to asialo GM1 that mediates the cellular response. Particularly worrisome is that most cell cultures contain BSA, and given that methylated BSA is an adjuvant for generating antibodies to asialo GM1, there is a strong possibility that BSA from culture medium bound to cells is the actual target of the cell-activating antibody.

Other bacterial factors that have also been shown to mediate adherence include the outer membrane porin, OprF⁹. Interestingly, expression of the OprF protein has been identified during growth in CF mucus¹⁷⁴ indicating the possibility of its availability to act as a bacterial ligand mediating attachment in this environment.

4.2. Adherence to other Non-Cellular Substrates

In addition to the potential of *P. aeruginosa* to bind to host cells and mucins, there are numerous reports indicating binding to cell-associated, extracellular or matrix proteins. These include fibronectin^{34, 153, 172} laminin, collagen and other components of the extracellular matrix³⁰, surfactant proteins^{102, 110, 133}, and heparin-sulfate proteoglycans^{20, 125}. The same bacterial adhesins implicated in binding directly to cells—pili, flagella, lipopolysaccharide (LPS), and alginate—have been implicated in binding to these structures. The importance of these interactions is poorly understood in most cases. While some of the earliest studies on *P. aeruginosa* interactions with host tissues implicated fibronectin as a receptor¹⁷² and pili as a ligand¹⁷¹ it has also been reported that fibronectin stimulates macrophages for nonopsonic phagocytosis of *P. aeruginosa*^{84, 86} and this host protective response is also mediated by pili. Additionally flagella are needed for nonopsonic phagocytosis of *P. aeruginosa* by macrophages and PMN¹⁰⁹. Surfactant proteins A and D bind to LPS and

opsonize *P. aeruginosa* for phagocytosis by alveolar macrophages^{110, 133}. Again these findings show how the same interaction of *P. aeruginosa* with the host could lead to either resistance to infection by recognition of LPS, pili, and flagella as targets for phagocytosis or to maintenance of the organism in or on a mucosal surface via binding to host tissues. Obviously the outcome depends on many other host and bacterial factors and one cannot simply predict from a single observation of the requirement of a bacterial factor for virulence if the same factor may not also serve as a target for host-mediated resistance to infection.

Heparin-sulfate components of host mucosal surfaces such as syndecans were also thought to be targets for bacterial binding^{20, 125}, but their role in infection was somewhat unexpected^{117, 118}. Syndecans are a family of at least four related compounds, composed of a protein core with heparin-sulfate substituents that are shed from the cell surface in response to tissue injury. Syndecan shedding in response to infection was originally thought to be a means to entrap bacteria and promote their clearance by such means as mucociliary clearance. Park *et al.* identified the LasA protease of *P. aeruginosa* as an inducer of syndecan shedding¹¹⁸ but surprisingly found that syndecan-1-deficient mice, expected to be more susceptible to infection, were in fact more resistant¹¹⁷. Wild-type mice treated with a reagent to inhibit syndecan shedding were more resistant to infection than untreated mice. In other words, the bacteria took advantage of the shed syndecans to promote pathology and disease instead of the syndecans binding the bacteria to promote their clearance. This finding again illustrates how simple concepts of host–bacterial binding are unlikely to be always true or applicable in a standardized fashion and in some instances binding of bacteria to host cells or components might promote host resistance to infection or could also be important to bacterial virulence and production of pathology and disease.

5. INTERNALIZATION

Another component of the *P. aeruginosa*–host cell interaction that has received a lot of attention is bacterial internalization or invasion^{38–40, 46, 47, 66}. Again the implications of this interaction for pathogenesis may vary widely depending on the situation under study. Fleiszig *et al.*⁴⁹ first showed that *P. aeruginosa* invades corneal epithelial cells during murine infection and subsequently showed the same phenomenon occurs with cultured cells *in vitro*⁵⁰. Pier *et al.*¹²² showed that cultured airway epithelial cells from CF patients were deficient in their ability to internalize *P. aeruginosa* and showed in neonatal mice that inhibiting internalization lead to increases in bacterial burdens and lethality^{121, 122}. They proposed one component of host resistance to infection

was desquamation of internalized bacteria by epithelial cells. However, internalization of bacteria by epithelial cells was found to promote infection in a model of corneal ulcerative keratitis¹⁷⁵, the difference being the localization of the infected airway epithelial cells to the mucosal surface from which they could desquamate vs the buried location of the infected corneal epithelial cells which were at the bottom of the 5–6 cell layer thick cornea. In the corneal model access to the deeper layer of cells is provided by a scratch injury⁴⁹. Of note, the proportion of bacteria within epithelial cells, when measured either in cell culture^{49, 121, 122} or in vivo^{121, 150, 175} is usually quite low when either a high multiplicity or a high level of infection is used, respectively. This suggests that internalization and clearance via desquamation may be more operative in situations such as low level infection acquired from the environment where only a few cells of *P. aeruginosa* are encountered that make it to an epithelial cell surface. When infectious doses are higher, desquamation may not contribute significantly to clearance, but resistance may nonetheless be high as epithelial cell induced signaling to the innate immune system may be a highly efficient back up system.

5.1. Invasion vs Cytotoxicity

In her investigations on corneal cell responses to *P. aeruginosa*, Fleiszig and colleagues⁵¹ found some clinical isolates did not invade cells but instead caused cytotoxicity. Thus, strains of *P. aeruginosa* can be classified as either invasive or cytotoxic; the invasive potential was found to inversely correlate with the ability of these bacteria to kill cells. Cytotoxicity is now known to depend on secretion of the type III protein ExoU⁷¹. Recent studies have shown that this toxin can kill *Saccharomyces cerevisiae*¹³⁰ and functions as a lipase, or actually a phospholipase, that requires a host cell co-factor for activity¹⁴⁴. This cytotoxin has been found to induce apoptosis in both macrophages and epithelial cells⁷⁰. But as mentioned earlier, although ExoU clearly enhances virulence when it is expressed, it is not present in many clinical isolates, indicating sufficient virulence among most invasive strains in the absence of ExoU production.

Genetic analyses have identified some of the factors involved in cytotoxicity. When normally cytotoxic strains, such as PA103, are genetically engineered to be defective in a gene encoding a regulator of type III secretion (*exsA*) they are more readily internalized into epithelial cells. However, mutation of this gene had no effect on internalization of the non-cytotoxic strain PAO1⁴⁸, indicating it was loss of the cytotoxic phenotype and not a gain in internalization capacity that occurred in the *exsA* mutant PA103 strain. Subsequent studies have shown that two type III secreted effectors, ExoS and

ExoT, act as anti-internalization factors. Thus, when these proteins are not translocated to the eukaryotic cells, increased uptake occurs. Internalization depends on specific actin cytoskeletal rearrangements and the type III secreted protein ExoS and ExoT disrupt this function. Both ExoS and ExoT have two domains: The N-terminal regions have Rho-GAP activity and the C-terminal regions have ADP-ribosyltransferase activity. The RhoGAP domain of ExoT and ExoS causes actin disruption through the inactivation of Rho, Rac, CDC42, and RalA^{55, 58, 83}. This function has been implicated in inhibition of phagocytosis in epithelial cells and macrophages^{57, 90}. The C-terminal domain of ExoS modifies Ras and Ras-like proteins by ADP-ribosylation¹¹⁹. This activity requires host cell 14-3-3 proteins for this function⁵⁶. The C-terminal domain in ExoT ADP ribosylates Crk-I and Crk-II, which may also have a role in the anti-internalization effect of ExoT¹⁵⁸. As both of these processes have been associated with virulence^{73, 108, 121, 22} the ability of ExoS and ExoT to inhibit cellular uptake of *P. aeruginosa* is likely key to some of the organism's virulence. However, different cell lines have different susceptibilities to the effects of these exoproteins^{23, 137} so choice of the proper cell line for study is critical and likely mirrors the role of these different factors at distinct sites of infection.

5.2. Bacterial Ligands for Internalization

To determine the bacterial factors required for internalization, strains with mutations in various implicated virulence factors have been tested in tissue culture models of infection. Previous studies noted that LPS rough strains of *P. aeruginosa* that do not contain O-polysaccharide side chains and are commonly isolated from chronically infected CF patients were internalized less well compared to LPS smooth strains. Addition of purified complete-core oligosaccharide to internalization assays inhibited bacterial uptake¹²². The complete core oligosaccharide portion of the LPS was found to be required for internalization and LPS was found to bind specifically to CFTR¹²¹. A recent report on the structure of the complete core oligosaccharide from a CF isolate, strain 2192, failed to identify any major changes in its structure compared to that found in wild-type, invasive strains⁸⁷. However, the outer core sugars were acetylated in a non-stoichiometric manner, which has not been previously observed in the LPS of non-CF isolates. The presence of the acetate groups may be important for the poor internalization of CF strains by epithelial cells. Perhaps more relevant to defining the LPS core structure that binds to CFTR have been the findings that *P. aeruginosa* produces two oligosaccharide cores, one of which accepts the O side chain and the other does not^{17, 87, 140, 141}. Although the CF isolate produced both of these variants, the one able to accept

O side chains was not substituted, as this strain is LPS-rough. This raises the possibility that the CFTR binding domain is the LPS outer core that is usually substituted with the O-antigen. However, when this core oligosaccharide glycoform is not substituted with O-antigen, as in CF isolates, it cannot interact with CFTR.

In another look at the role of CFTR in *P. aeruginosa* uptake, Chroneos *et al.*²² found no difference in virulence of *P. aeruginosa* in transgenic mice lacking CFTR expression in the lungs, wild-type mice, or mice over-expressing human CFTR in the lung and concluded that there was no correlation between CFTR expression and host resistance to infection. However, these workers used strain FRD1 for their in vivo studies, which, because it is LPS-rough, would be poorly internalized even in wild-type cells. In fact, Schroeder *et al.* confirmed this prediction¹⁵⁰ and showed that the effect of CFTR-dependent epithelial cell clearance is related to expression of a smooth LPS with strains having the capacity to interact with CFTR.

The role of flagella and pili in host cell uptake of *P. aeruginosa* has also received some attention^{143, 160}. However, a concern with many of these studies is that they have reported an effect of pili and flagella on both adherence and internalization but since these structures contribute to bacterial cell motility, the mutants are less able to move towards epithelial cells in a directed manner. As such, one cannot separate a lack of adherence or internalization from a loss of motility or a loss of a ligand for a cellular receptor^{143, 160}. Indeed, it was found early on in one study that if non-motile *P. aeruginosa* were lightly centrifuged onto epithelial cell monolayers, the apparent reduced uptake of these mutants was no longer seen⁵⁰.

In contrast, results with a *flhA* mutant, suggested that expression of this factor was involved in *P. aeruginosa* adherence and internalization by cultured rabbit corneal epithelial cells. These mutants were defective in at least some aspect of flagella synthesis as they were non-motile, similar to a *fliC* mutant. Centrifugation of these mutants onto rabbit corneal epithelial cells did not affect internalization, and, in fact, these mutants actually adhered better to corneal cells⁴⁴. When the levels of internalization (as a percent of adherence) were measured, it was found that these mutants had an internalization defect. However once inside the cells the *flhA* mutant were able to survive as well as the wild-type strain⁴⁴. Overall, some *P. aeruginosa* factor dependent on an intact *flhA* gene is needed for optimal bacterial uptake by some cells.

5.3. Host Receptors for Internalization

Two major receptors on epithelial cells have been proposed to mediate internalization of *P. aeruginosa*: CFTR¹²¹ (Figure 3) and asialo GM1²⁵. It has

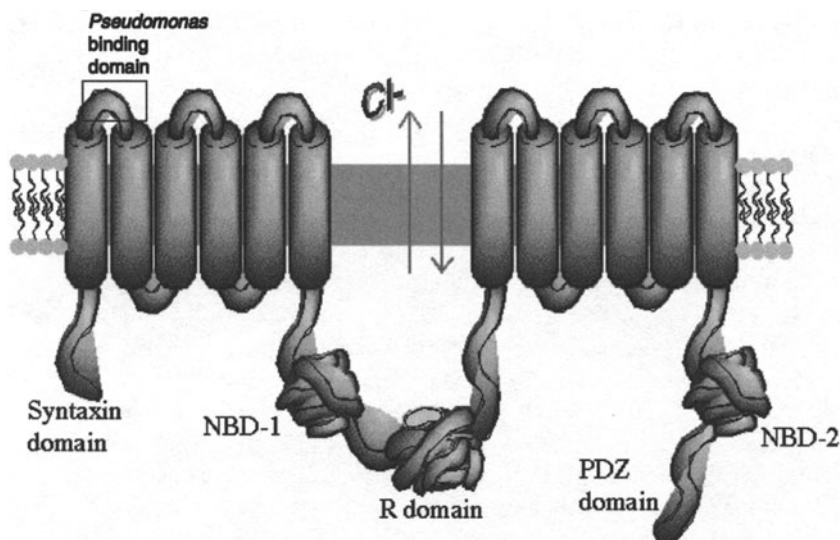


Figure 3. Schematic depiction of CFTR with its associated domains and functions. Two nucleotide binding domains (NBD) bind ATP and hydrolyze it to activate and regulate the channel. The syntaxin binding domain at the amino terminus and the PDZ binding domain at the carboxy terminus are involved in many processes regulating CFTR function and trafficking. The R or regulatory domain is involved in gating of chloride and likely bicarbonate ions through the channel pore. The *P. aeruginosa* binding domain in the first predicted extracellular loop is also indicated.

been proposed that CFTR functions as a type of pattern recognition molecule similar to the toll-like receptors (tlrs) but with specificity for a limited range of bacteria¹⁴⁹. CFTR binds to *P. aeruginosa* outer core LPS¹²¹, removes the LPS from the bacterial membrane, and the epithelial cell responds with a rapid and robust NF- κ B nuclear translocation¹⁴⁹. Amino acids 108–117 have been identified as the specific portion of CFTR that binds *P. aeruginosa*¹²¹. Esen *et al.*³⁸ also showed that CFTR serves as a ligand for internalization of *P. aeruginosa* into epithelial cells, and inhibition of this interaction with CFTR peptide 108–117 blocked tyrosine kinase-mediated epithelial cell signaling. The need for functional CFTR to properly respond to wild-type *P. aeruginosa* LPS leading to nuclear translocation of NF- κ B in both human epithelial cells in culture and in mouse lung tissues provides compelling evidence for this response being a key component of innate immunity to *P. aeruginosa* infection that is dysfunctional or absent in CF patients.

However, as noted above, CFTR is expressed in the corneal epithelium and similarly serves as an internalization receptor for *P. aeruginosa*, but in this

case leads to pathology and disease. In contrast to transgenic CF mice that are highly susceptible to both acute and chronic *P. aeruginosa* lung infections^{24, 61, 150, 165} in comparison to wild-type mice, transgenic CF mice are more resistant to *P. aeruginosa* corneal infection¹⁷⁵. The differences in epithelial cell location—surface vs subsurface—appear to underlie the opposing outcomes of *P. aeruginosa* lung and eye infections in the context of CFTR-mediated epithelial cell ingestion.

Asialo GM1 has been reported by many investigators to serve as a ligand for *P. aeruginosa*, and Comolli *et al.*²⁵ also showed that binding of *P. aeruginosa* strain PA103 to asialo GM1 was involved in internalization of non-cytotoxic mutants of this strain. But the same problems apply to the validity of the conclusion regarding a role for asialo GM1 in internalization that occur with the studies on bacterial binding to asialo GM1. Specifically, PA103 and perhaps a few other laboratory strains are the only ones with some increased binding to asialo GM1 whereas most clinical isolates show no preference for this cellular receptor¹⁵¹. No *in vivo* studies have been performed showing that blocking *P. aeruginosa* binding to asialo GM1 affects the outcome of this pathologic process.

5.4. Bacterial Survival Inside of Epithelial Cells

Once internalized, the survival of *P. aeruginosa* within the epithelial cell can obviously affect whether host immunity will dominate by killing the organism, or bacterial virulence may become manifest due to survival, proliferation, and elaboration of toxic factors. Interestingly, Evans *et al.*³⁹ found that a complete LPS core and O-antigen is required for survival of two non-cytotoxic *P. aeruginosa* strains after internalization into corneal epithelial cells. At early time points of infection, there was no apparent difference in the levels of invasion between a wild-type strain and an LPS-rough mutant. However the wild-type strain replicated in these cells while the LPS-rough mutants that had invaded these cells were all killed during the 3–4 hr time frame of this experiment. Thus, in addition to its role in serum resistance and internalization, a complete LPS has a role in survival even within epithelial cells.

Another gene that appears to have a role in intracellular survival is *dsbA*. DsbA is a periplasmic protein that catalyzes the folding and assembly of secreted proteins. This protein has many distinct roles important for virulence. DsbA appears to be required for type III secretion in PA103 as well as intracellular survival in HeLa cells⁶⁷. A *dsbA* mutant also showed a defect in binding to HeLa cells compared to the wild-type strain and a defect in twitching motility. While the large effects of missing DsbA on *P. aeruginosa* preclude attributing lack of survival in epithelial cells to a specific factor, it does illustrate that when measuring internalization, combinations of uptake,

survival, and proliferation will all contribute to the final amount of bacterial cells deemed to be internalized.

6. HOST CELL RESPONSES TO ADHERENCE AND INTERNALIZATION

The cellular events following adherence and internalization of *P. aeruginosa* to epithelial cells have been analyzed. The read outs have been varied, including measurement of NF- κ B nuclear translocation, induction of IL-8 as a marker of inflammation, and activation of genes or proteins involved in epithelial cell signaling such as tyrosine kinases.

6.1. Signaling Events and Cellular Responses Associated with Bacterial Cell Adherence and Invasion

Schroeder *et al.*¹⁴⁹ reported that CFTR could actually extract LPS from the outer membrane of *P. aeruginosa* and this activity was associated with rapid nuclear translocation of NF- κ B. Such activation did not happen with human cells lacking wild-type CFTR nor in lung cells of *P. aeruginosa*-infected transgenic CF mice. Furthermore, NF- κ B nuclear translocation does not occur with *E. coli* nor with *P. aeruginosa* strains lacking the complete outer core oligosaccharide that binds to CFTR¹⁴⁹. The NF- κ B translocation started by 5 min, peaked around 15 min and was over by 45 min. They proposed that this rapid response is critical for orchestrating *P. aeruginosa* clearance from the lung. Additional work reported in an abstract (Reiniger N., Ichikawa J., Lory S., Pier G.B., CFTR-dependent epithelial cell gene expression in response to *P. aeruginosa*. Abstracts of the 102nd General Meeting of the American Society for Microbiology, 2002. Abstract D-76) has shown that there is a CFTR-dependent activation of NF- κ B gene transcription following *P. aeruginosa* infection of human airway epithelial cells expressing wild-type CFTR that is not found in cells with mutant alleles of *CFTR*. This work indicates that another basis for the hypersusceptibility of CF patients to *P. aeruginosa* infection is an inability to activate host inflammatory responses properly following *P. aeruginosa* lung infection.

Other investigators have shown that epithelial cell uptake of *P. aeruginosa* requires Src family kinases and calcium-calmodulin activity⁴⁰. Similar studies noted the requirement of activation of the Src-like tyrosine kinases p59Fyn and p60Src and the consequent tyrosine phosphorylation of several eukaryotic proteins for efficient bacterial uptake³⁸. Inhibition of Src-like tyrosine kinases resulted in inhibition of *P. aeruginosa* internalization, but not adherence to

cells. In fact, adherence was increased, suggesting that lack of removal of cell-surface bound bacteria contributed to the overall finding of increased adherence. This study also showed that inhibition of *P. aeruginosa* binding to CFTR by use of the specific peptide to which *P. aeruginosa* binds prevented the activation of Src and Fyn³⁸. This work supports the key role of CFTR-dependent signaling in response to *P. aeruginosa* as instrumental in wild-type resistance to infection.

In another study it was found that mutation of Csk, which encodes the C-terminal Src kinase, was found to reduce internalization of *P. aeruginosa* into fibroblasts⁴¹. As Csk negatively regulates Src tyrosine kinase activity and therefore mutations in Csk increase the activity of Src and Fyn, based on the results of Esen *et al.* it might have been anticipated that this mutation would result in increased internalization. Whether this is a cell type-specific process or Csk regulates other pathways required for internalization is not clear. However, what it likely indicates is that a proper, coordinated, response to *P. aeruginosa* infection is needed by the epithelial cell and disruptions in both positive and negative regulators could alter the overall outcome observed.

Other host proteins that are involved in the internalization process include MAP kinases including MEK and ERK⁴². Studies using an invasive *P. aeruginosa* strain from a corneal infection and corneal epithelial cells showed that a MEK-specific inhibitor, PD98059 and U0126 inhibited *P. aeruginosa* internalization, but not cellular association. A *P. aeruginosa* strain with a mutation in the *flhA* gene that was ingested less well than a wild-type strain induced lower protein phosphorylation responses⁴². Phosphorylation of ERK was also noted to be induced by *P. aeruginosa* elastase in both A549 lung epithelial cells and rabbit alveolar type II epithelial cells⁸.

Some specific bacterial components for epithelial cell activation have been identified. Wild-type strain PAO1, but not PAO1 lacking pili, stimulated p38 and ERK1.2 activity resulting in NF- κ B nuclear translocation and IL-8 secretion. This was reported to be mimicked by ligation of asialo GM1 receptors in a calcium-dependent manner¹³². However, as noted earlier, the use of a commercially available antibody to asialo GM1 which has specificities to both bacterial and epithelial cell antigens in addition to asialo GM1 cannot be considered definitive enough to conclude that these pathways are activated by binding asialo GM1.

P. aeruginosa adherence appears to alter gene expression in the host cells⁷⁸. Changes in gene expression were followed in A549 cells after infection with strain PAK or strain PAK lacking pili. A number of genes were noted to have increased transcription in response to infection with the wild-type strain that were not changed as much with infection by the non-piliated mutant. One gene followed in detail was that encoding IRF-1 (interferon regulatory factor 1), a transcription factor, whose expression has been shown to lead to the

activation or repression of target genes. The genes downstream of IRF-1 were not identified in this study, however others have noted that inducible nitric oxide synthase expression is induced and secretory leukocyte protease inhibitor expression is repressed by IRF-1.

It has also been shown that *P. aeruginosa* binding to epithelial cells stimulates mucin secretion^{98, 99}. However, this has not been evaluated in regard to a role for CFTR in this process. In cells with wild-type CFTR, it was found that a Src-dependent Ras-MAPK-pp90rsk pathway lead to NF- κ B nuclear translocation and mucin gene transcription. What is not clear is whether this response is part of the normal host physiology involved in stimulating mucociliary clearance, or in the pathology of CF lung disease where dehydrated mucus can serve as a nidus for bacterial infection^{13, 173}.

McNamara *et al.*¹¹¹ also reported that flagella binding to asialo GM1 induced mucin gene transcription via secretion of ATP. In other systems, flagellin stimulates matrilysin expression¹⁰⁶. However the role of this induction in the CF infectious process is not clear as strains from these infections often lack flagella¹⁰⁸.

6.2. IL-8 and Inflammatory Markers in Cellular Responses to *P. aeruginosa*

In many cases the cellular events following adherence and internalization of *P. aeruginosa* by epithelial cells have been monitored by evaluation of the induction of IL-8 as a marker of inflammation. In particular, a role for CFTR in this process has been of interest, as some clinical and laboratory studies suggest that CF patients may have a predisposition to lung inflammation in the absence of detectable infection^{85, 159, 162}. However, other studies suggest that with proper evaluations and intensive culturing combined with serologic analysis of antibody responses to *P. aeruginosa*, many young CF patients actually become infected, at least transiently, with *P. aeruginosa* at a very young age^{16, 168}. As far as inflammatory markers as part of the cellular responses to *P. aeruginosa* is concerned, this is a confusing area, as some studies have shown that CF cells spontaneously produce IL-8 even in the absence of *P. aeruginosa* stimulation^{35, 132}. Others have disputed this finding, using human cells and cells from transgenic CF mice^{124, 147} and do not find spontaneous NF- κ B nuclear translocation in CF airway cells¹⁴⁹. It appears that spontaneous IL-8 secretion is really very cell line dependent, and not a regular feature of all CF cell lines¹ and is not usually found in intact tissues from CF mice or primary cultures of cells from humans. But many studies do find IL-8 and similar inflammatory cytokines made by cells in response to *P. aeruginosa*, but here again the caveat applies as to whether this is part of the normal host response that clears *P. aeruginosa* and is defective in CF¹⁴⁹ or part of the pathologic response contributing to inflammatory damage.

Likely both are true, with early inflammatory responses made in a CFTR-dependent manner being needed for high level resistance to *P. aeruginosa* infection, but when this fails, as it does in CF and perhaps is just overwhelmed in acute *P. aeruginosa* infection, then over-zealous inflammatory responses mediated by such factors as IL-8^{62, 152} become pathologic.

Some specific *P. aeruginosa* factors have been proposed to promote induction of IL-8 secretion. Bacterial nitrate reductase from *P. aeruginosa* induces IL-8 expression and production in respiratory cells¹¹². The autoinducer 3-O-C12-HSL, a quorum sensing molecule known to be important for binding and activating transcription of a number of *P. aeruginosa* virulence genes, has been shown to activate IL-8 production in human lung fibroblasts and epithelial cells¹⁵⁴. In addition, this molecule has been found to induce an inflammatory response and cause tissue destruction of mouse skin cells¹⁵⁵. Overall the proper balance of appropriate inflammation to eliminate infection and inappropriate inflammation promoting tissue pathology is regulated to a large degree by epithelial cells responding to *P. aeruginosa*, emphasizing the importance of this interaction in the disease process.

7. APOPTOSIS

Apoptosis or programmed cell death is often found as a component of cellular responses to *P. aeruginosa* (Figure 4). In general this is viewed as a better outcome to infection as compared with necrosis or oncosis, as apoptosis

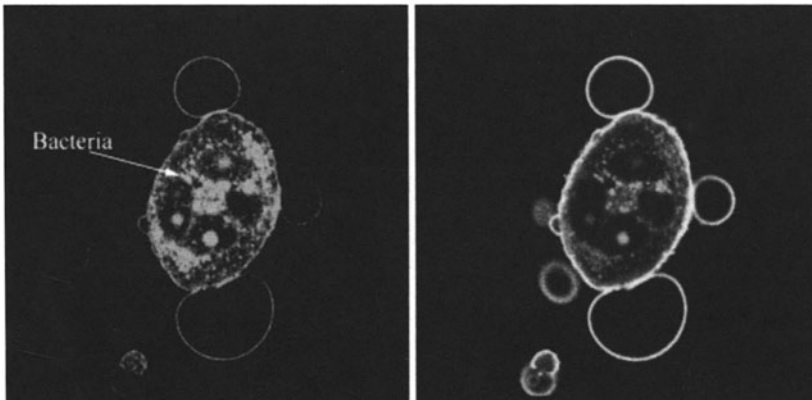


Figure 4. Demonstration of a cell with wild-type CFTR undergoing apoptosis. Stained *P. aeruginosa* are visualized on the left hand panel while the right hand panel shows the stained plasma membranes and apoptotic blebs being formed. Membrane is stained to visualize the annexin 5 marker of apoptosis.

results in much less inflammatory damage to host tissues. However, inducing host cells involved in immunity to undergo apoptosis could be beneficial to a pathogen, and avoiding additional inflammation may be of further benefit in escaping the effects of host defenses. Porins have been reported to induce apoptosis in a cell line derived from rat seminal vesicles¹⁵, but most studies have focused on the role of the type III secretion system in induction of apoptosis in host cells.

Type III secretion mutants have been found to be defective in inducing apoptosis of macrophages and epithelial cells^{69, 81}. This effect was independent of exotoxin A and/or ExoU, but does require an intact type III secretory system. Interestingly, HeLa but not MDCK cells were susceptible to this type of killing. Mutant strains of *P. aeruginosa* deficient in ExoS failed to induce apoptosis and this was found to be dependent on the ADP-ribosylation activity of ExoS⁸². Other workers have noted that another type of cell death, oncosis, is also dependent on type III secretion but independent of ExoU²⁸. The exact implication of this phenomenon is unclear, but necrotic or oncotoc cells tend to have much more inflammatory capabilities than apoptotic cells. This could be bad for the organism as enhanced inflammation may promote clearance, or it could be bad for the host as enhanced inflammation can lead to irreversible tissue destruction and loss of function.

The role of CFTR in the ability of a cell to undergo apoptosis has been studied by several groups. Rajan *et al.*¹³¹ reported no effect of CFTR in this process, but these investigators did not look at a variety of time points to see if there were differences in the kinetics. In contrast, Cannon *et al.*¹⁹, recently found a moderate but reproducible effect of CFTR on the induction of apoptosis following exposure to *P. aeruginosa*, wherein cells with wild-type CFTR underwent a more rapid apoptotic response. Interestingly, internalization of *P. aeruginosa* was not needed for induction of apoptosis, as inhibition of bacterial uptake by a low dose of cytochalasin did not affect induction of CFTR-dependent apoptosis. This finding suggests that more rapid resolution of the inflammatory response to *P. aeruginosa* occurs in a wild-type situation. In support of this view, studies by Gulbins and colleagues first showed a clear role for apoptosis in host resistance to *P. aeruginosa* infection⁶³. In this study mice defective in either Fas or Fas ligand (CD95 or CD95 ligand) were more susceptible to infection.

In addressing both CFTR dependence and apoptosis as components of resistance to *P. aeruginosa* infection, Grassme *et al.*⁶² reported that *P. aeruginosa* infection of either human nasal epithelial cells or murine tracheal epithelial cells induced reorganization of the GM1 sphingolipid in cell membranes. GM1 is a component of lipid rafts, and disruption of this sphingolipid-enriched platform prevented *P. aeruginosa*-induced apoptosis in the cultured cells. These authors showed using confocal microscopy that both CFTR and

CD95^{63, 64}, which induce internalization and apoptosis respectively, were able to localize to these rafts, but more robust demonstrations of raft localization by CFTR and CD95 by techniques such as showing CFTR or CD95 in triton-insoluble lipid raft fractions was not reported⁶². As part of this process it was documented that *P. aeruginosa* activated acid sphingomyelinase that was followed by translocation of this enzyme from vesicles to the extracellular leaflet of the cell membrane. Movement to the plasma membrane was also correlated with the formation of ceramide clusters in this membrane. Without this ceramide-mediated raft modification there was a failure to internalize *P. aeruginosa*. Acid sphingomyelinase defective cells also released more IL-1 β and mice with defects in this enzyme were much more susceptible to the lethal effects of *P. aeruginosa*. This result is similar to what has been found in IL-1 defective mice that also were to be more resistant to *P. aeruginosa* pneumonia than wild-type mice. Resistance to infection was reflected in a lower influx into the lung of neutrophils and with lowered cytokine and chemokine production. Therefore, the interference with CFTR-dependent cellular function and apoptosis in the acid sphingomyelinase-deficient mice supports the idea that normal, CFTR-dependent cellular internalization and subsequent epithelial cell responses leading to eventual apoptosis are associated with clearing *P. aeruginosa* infections. In situations where this is defective, such as CF, a state of increased susceptibility to *P. aeruginosa* infection ensues.

That CFTR epithelial expression is critical to high-level resistance to *P. aeruginosa* infection was elegantly demonstrated by Oceandy *et al.*¹¹⁵ in a transgenic mouse system. Oceandy *et al.*¹¹⁵ took transgenic mice homozygous for the G551D alleles of CFTR, which is associated with high susceptibility to *P. aeruginosa* infection, and showed that if they directed expression of wild-type CFTR to either the alveolar macrophages or epithelium only the mice with wild-type CFTR in the epithelium regained resistance to *P. aeruginosa* infection comparable to that of mice with endogenous wild-type CFTR.

8. INTERACTIONS WITH MACROPHAGES

Macrophages in the lower airways are often considered one of the first lines of defense against *P. aeruginosa* in this environment, but a study by Speert and colleagues in mice found no role for alveolar macrophages in resistance to *P. aeruginosa* infection²¹. Thus, other studies that found effects of *P. aeruginosa* on such cells may reflect in vitro findings that may not be particularly relevant to the pathogenesis of infection. In one such study, using a macrophage cell line⁹⁶, there was less phagocytosis of *P. aeruginosa* PAO1 in cells expressing dominant negative Rac or CDC42 proteins, as well as in cells expressing a Rac1/CDC42-specific GTPase domain, Chim-GAP. However,

inhibition of these G-protein linked interactions did not affect nuclear translocation of the p65 subunit of NF- κ B or of NF- κ B-dependent expression of inducible nitric-oxide synthase, COX-2, or TNF- α . Isogenic mutants lacking either pili or flagella or both are poorly phagocytosed and ingested by macrophages^{108, 109}, but lack of these bacterial structures does not effect the expression of the proinflammatory genes from infected macrophages. The conclusion of this study was that pro-inflammatory protein expression is not critically linked to phagocytosis of *P. aeruginosa* by alveolar macrophages. The lipid A moiety of LPS likely contributes to the innate immune response to *P. aeruginosa*. In most Gram-negative bacteria, lipid A interacts with the toll-like receptor 4 (TLR4) on macrophages and similar cells. In *P. aeruginosa*, the major form of lipid A produced in vitro is less inflammatory than enterobacterial LPS but it has been noted that the structure of lipid A can vary depending on the strain, source of infection, and culture conditions. For example, a *P. aeruginosa* strain from a CF patient has been found to have a modified lipid A structure containing increased amounts of palmitate and aminoarabinose³⁷. This alteration was correlated with increased resistance to antimicrobial peptides and enhanced induction of IL-8 from an endothelial cell line. These findings suggest that whereas *P. aeruginosa* LPS isolated in vitro from non-CF strains generally elicits a muted response in comparison to enterobacterial LPS, a structurally modified LPS produced during lung infection of CF patients can potentially elicit a more pronounced inflammatory response. Further analysis has revealed that the LPS glycoform produced during chronic lung infection was more active at stimulating TNF- α and IL-8 release from a human macrophage cell line, but activation of pro-inflammatory mediators in a murine macrophage cell line was similar when this LPS glycoform was compared with that of a laboratory strain of *P. aeruginosa*. These authors uncovered differences in the TLR4 molecules from humans and mice that were responsible for the specific recognition of these two different *P. aeruginosa* LPS molecules⁶⁸. Thus, a highly specific and adaptive change in the bacterial LPS and subsequent adaptive host responses may be manifest in the lung of chronically infected CF patients, leading to increased inflammation and increased bacterial resistance to host immune effectors. Aside from LPS, the alginate of *P. aeruginosa* has been shown to stimulate cytokine production¹¹⁶. Interestingly the amount and percentage of mannuronic acids (one of the components of alginate) was correlated with this activity. Induction of TNF- α by monocytes was blocked with antibodies to TLR2 and TLR4, indicating the importance of both these receptors in recognition of alginate. Macrophages from *tlr4* mutant mice were entirely non-responsive to a preparation of polymannuronic acid, whereas macrophages from *tlr2* knock out mice were partially responsive, indicating that TLR4 may have a more dominant role in this interaction⁵⁴. Overall, many *P. aeruginosa* factors, including LPS, alginate,

and lipoproteins likely provoke both the desirable, protective inflammatory response and the undesirable, pathologic inflammatory response that occurs during *P. aeruginosa* infections, with the importance for the host dependent on the timing, intensity, and kinetics of the inflammatory molecules produced.

9. INTERACTIONS WITH MAST CELLS

Under appreciated in the host response to *P. aeruginosa* is the role of the mast cell. These cells occur in high numbers in the lung and in the skin, where their reaction to a *P. aeruginosa* infection that can occur subsequent to a burn injury could be important. Mast cells are important as sentinels in tissues monitoring disruption of normal function. Mast cells rapidly respond to infectious agents to coordinate host defenses. However, only a minimal amount of information is known about the role of mast cells in the pathogenesis of *P. aeruginosa* infection, and this information is based mostly on descriptive studies that suggest that mast cells respond vigorously to *P. aeruginosa* in a manner that is consequential for both host resistance and the development of the pathologic process. Bergmann *et al.*¹² showed *P. aeruginosa* stimulated histamine release from rat mast cells. Various mast cells from rodents and humans have been shown to produce IL-1 and IL-6 in response to *P. aeruginosa*^{14, 103}, with the former inducing PMN transmigration across the endothelium and potentially bringing the PMN to the basal side of the epithelium where they may further migrate to the apical side of the mucosal surface and contribute to host resistance to infection. However, in chronic infection the continued mast cell production of these inflammatory cytokines could be detrimental. In response to *P. aeruginosa*, mast cells also produced the chemokine CCL20, also known as macrophage inflammatory protein 3 α , which play a role in recruiting dendritic cells and T cells to tissues to initiate acquired immune responses. While it is not known if there is an effect of CFTR expression on these mast cell functions, and whether or not the inability of the CF patients to mount an effective acquired immune response to *P. aeruginosa* is related to mast cell dysfunction, the role of these cells should not be ignored when considering *P. aeruginosa* interactions with immune-effector cells, particularly when extrapolating findings from in vitro cell cultures to in vivo situations.

10. THE ROLE OF PMN IN HOST RESISTANCE TO INFECTION

For many years clinical studies have shown that neutropenic patients have a very high susceptibility to *P. aeruginosa* infection³⁶. A clear role for PMN in resistance to *P. aeruginosa* infection is well appreciated. Indeed,

studies in neutropenic animals show that strains of *P. aeruginosa* that require 10^7 to $>10^8$ cfu to cause a lung or systemic infection can be lethal when as few as 10 cfu are given to a neutropenic animal^{27, 129}. These findings raise a critical issue—are PMNs really the critically important effector cell in innate immunity to *P. aeruginosa*? While epithelial cells clearly can produce factors to coordinate PMN migration, activation, and phagocytosis of *P. aeruginosa*, in the absence of PMN it appears, at least from animal studies, that no other innate immune effectors are sufficiently potent to control infection. Thus molecules like defensins produced by epithelial cells may be only of marginal value in resistance to *P. aeruginosa* infection, although defensins in the PMN granules may be critical. One caveat for these studies is that neutropenia is usually produced by administering the cytotoxic drug cyclophosphamide, a drug known to effect rapidly dividing cells and epithelial cells. Hirikata *et al.*⁷⁴ showed that mice treated with cyclophosphamide developed a higher rate of *P. aeruginosa* bacteremia than did mice treated with other cytotoxic drugs. Thus, there may be some effects of cyclophosphamide on host resistance to *P. aeruginosa* beyond its ability to deplete PMN levels. Overall, studies using specific depletion of PMN with a neutrophil-specific antibody will be needed before a definitive conclusion can be drawn that PMN are the essential mediators of immunity to *P. aeruginosa* infection.

11. TRANSCYTOSIS

Movement across epithelial cell membranes is a prerequisite for the development of septicemia and dissemination. However, how this occurs in vivo is not well understood. An in vitro model of *P. aeruginosa* transmigration across a polarized epithelial cell monolayer using bacteria applied to the apical surface of cells grown on transwell filters has been used in some recent studies. Movement of bacteria across the epithelium is monitored by following bacterial transport to the basal side of the cells. A study by Hirakata *et al.*⁷³ revealed that transcytosis was correlated with the original site of recovery of organisms from human infections, with blood isolates significantly better able to penetrate the monolayer compared with respiratory isolates. Epithelial monolayer penetration was independent of cytotoxic activity⁷³ although cytotoxic strains produced a decrease in TER as they killed the epithelial cells. However, it was not entirely clear if transcytosis occurred via migration through the epithelial cells or paracellularly between them. The finding that respiratory isolates of *P. aeruginosa* do not transcytose through the epithelium as well as blood isolates is important as it is known in human *P. aeruginosa* pneumonia that bacteremia is a rare complication, suggesting that many strains can remain confined to the lungs and cause severe pneumonia but have

a lessened capacity to disseminate. Further studies by this group showed that *P. aeruginosa* efflux pump mutants had a decreased ability to transmigrate through MDCK epithelial cells⁷⁵. The most dramatic result was obtained with a *mexAB-oprM* mutant that could not invade the cells or transmigrate across the epithelium and could not kill mice. Other mutants were intermediate in their effects. Supernatants from wild-type strains were able to restore the cellular invasiveness, suggesting that these efflux pumps secrete factors important for virulence.

12. CONCLUSIONS

Overall there is clearly a large effect of *P. aeruginosa* on the epithelial cells that the organism first encounters when it infects a mucosal surface. The bacterial–epithelial cell interaction critically impacts the outcome of the infection. As it is believed that exposure to *P. aeruginosa* in the environment is common and frequent, with aerosol exposure leading to deposition of bacterial cells in the respiratory tract, much of the focus of research investigators has been on airway epithelial cell responses to this organism. In addition, the high susceptibility of CF patients to *P. aeruginosa* infection clearly implicates a defect in the response of the lung to *P. aeruginosa* in these patients that is associated with lack of functional CFTR. Mouse studies show that CFTR expression in the airway epithelium is key to high-level innate immunity to *P. aeruginosa* lung infection¹¹⁵. However, studies of the molecular and cellular interactions of *P. aeruginosa* and epithelial cells, and the consequences of this interaction, have not led to any consensus about the basis for pathogenesis and host resistance to infection. Underlying these disparate findings are variables in the different investigator's systems for measuring bacterial–cell interactions, such as bacterial strains tested, cell lines used, whether the cells are polarized or not, and the integrity of reagents such as commercially-available antibody to asialo GM1. Also, correlative in vivo studies are lacking in many instances, yet there are now numerous, transgenic mouse lines with appropriate defects in receptors and signaling components that can be used to study the pathogenesis of *P. aeruginosa* infection to verify that in vitro effects are important in an in vivo setting.

Another important consideration is timing: *P. aeruginosa* interactions with cells is likely rapid, occurring very early on in the infectious process and determining whether the host will successfully fight off infection or the organism will overwhelm defenses and cause pathology, disease, and perhaps death. In a situation such as chronic infection, or even acute infection after several hours, ongoing responses of cells to bacteria and bacterial products will also be a key component of the pathogenic process, but usually at these time points

there is a complex mixture of cells responding to *P. aeruginosa*, making it difficult to ascribe a particular outcome to interactions with a single type of cell. For example, PMN, macrophages, mast cells, and epithelial cells can all produce inflammatory cytokines in response to *P. aeruginosa*, so once the infection has progressed to the point where these cells are activated by infection, individual contributions from different cells to the ongoing response may vary markedly and these responses will be critically influenced by the strain of *P. aeruginosa* being used.

Studies that rely principally on laboratory strains such as PAO1, PA103, and PAK may not yield results applicable to clinical isolates, and it is clear that even among some of these strains, such as PAO1, there is marked genetic and phenotypic variation between strains with this designation used by different investigators⁸⁸. Fresh clinical isolates from patients with the disease under study and appropriate to the question being asked need to be used to confirm results with laboratory strains. Considerations for whether one is looking at an early phase of the *P. aeruginosa*–host cell interaction, as might occur within minutes of infection and determine if the organism will be cleared or proceed to grow, infect, and ultimately induce pathology must be juxtaposed with effects that would occur during chronic infection. In early phases of infection, LPS-smooth, non-mucoid strains typical of those found in the environment are likely the most appropriate strains, whereas for chronic lung infections typical of CF, studies with LPS-rough, mucoid strains are going to be more informative about how host cells respond in this the disease. Also, the growth phase of the bacteria can affect the outcome, as was shown for studies on apoptosis wherein log-phase, but not stationary-phase bacteria induced apoptosis⁷⁷.

With all of these caveats it may be difficult to synthesize a scenario generally applicable to *P. aeruginosa*–host cell interactions and agreed to by most investigators. Multiple groups report a role for CFTR in binding, internalization, and responses of epithelial cells to *P. aeruginosa*, and multiple groups report binding and cellular responses due to interactions with asialo GM1, but there are also published data that do not support these findings. More generally accepted is the role of ExoU in cytotoxicity and its affect on cells. Where the biggest controversy lies is in the implications of the observed effects of *P. aeruginosa*–host cell interactions for host resistance and bacterial pathogenesis. Likely much of this is contextual—a given response made early on in infection may be essential for host resistance to infection, such as IL-1 β production⁴ whereas the continued production of IL-1 β when innate immunity is overwhelmed, such as following a high bacterial inoculum into the lung, is associated with pathology¹⁵². Early IL-8 production shortly following infection is likely protective, continued IL-8 production during chronic infection is likely pathologic.

Finally, it is almost inescapable that different cell lines, growth conditions, and quality of reagents used will impact the findings and will lead to

continued controversies over the effects *P. aeruginosa* has on cellular responses. Whenever feasible use of isogenic cell lines and bacterial strains is critical for looking at effects such as expression of CFTR or expression of bacterial virulence factors. Insuring that reagents are specific and have the reported activity is also essential. Overall, to make sense of the multitude of findings on *P. aeruginosa*–host cell interactions it will be necessary to vigorously define conditions, reagents, and context of experimental findings and to validate the findings using multiple bacterial strains, cell lines, and appropriate and specific reagents. It is critical to acknowledge that in vitro cultures and experimental conditions do not mimic the complexity of bacterial states and host tissues in vivo and thus it is critical to undertake experiments to confirm in vitro findings with appropriate in vivo studies, to the extent that is feasible. Exchange of reagents, cell lines, protocols, etc. among investigators with disparate findings to try and identify sources of disagreement will greatly aid in defining the true nature of *P. aeruginosa*–host cell interactions. There is much to be learned from studying *P. aeruginosa*–host cell interactions that will lead to better understanding of the disease process and ultimately development of appropriate therapies for patients, but care and concern for context are critical for conducting the relevant experiments and interpreting the results properly.

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THE BIOFILM LIFESTYLE OF PSEUDOMONADS

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1. INTRODUCTION

Although the growth of bacteria in planktonic culture has been the mainstay of microbiological technique from the time of Pasteur to the present, and has provided an increasingly accurate understanding of prokaryotic physiology and genetics, it is now clear that bacteria in natural environments (except for the open oceans) live in surface-associated communities, and that knowledge about this biofilm lifestyle is essential in order to understand bacterial biology¹². During the last 10 years there has been a rapidly increasing recognition of biofilms as a highly significant topic in microbiology with relevance to many important areas in modern society such as drinking water supply systems, industrial settings, waste water treatment, bioremediation, chronic bacterial infections, nosocomial infections, and dental plaque.

The pseudomonads, especially *Pseudomonas aeruginosa*, have been used extensively as model organisms for the study of biofilm formation, and many of the most important contributions to our understanding of biofilm development come from these studies. The present chapter is focused on biofilm formation by *P. aeruginosa*, *Pseudomonas putida*, and *Pseudomonas fluorescens* in *in vitro* model systems. In Sections 2, 3, and 4 we describe the biofilm developmental cycle as: Initial biofilm development, further biofilm development and maturation, and biofilm dissolution and dispersal. In Section 5 we describe diffusion, gradients, and physiological heterogeneity in biofilms, and finally in Section 6 we describe innate resistance of biofilms towards antimicrobials.

2. INITIAL BIOFILM DEVELOPMENT

Behaviour of *Pseudomonas* cells during initial biofilm development has been investigated microscopically in flow-cell set-ups, and the genetic elements involved in the initial phase of biofilm formation has been investigated by mutant analysis using high-throughput screening assays for biofilm formation.

2.1. Mechanisms Involved in Initial Biofilm Development

The processes involved in the initial phase of biofilm development include (a) transport of microbes to a surface, (b) attachment to the surface, and (c) formation of initial microcolonies.

2.1.1. Transport of Bacteria to the Substratum. In order for bacteria to reach a substratum prior to colonization they may have to overcome repulsive forces at the liquid–solid interface⁵⁸, which can be achieved by passive motility mediated by Brownian motion or vortex currents, or by active motility mediated by flagella rotation. Flagellum driven motility (or flagella *per se*, see below) has been reported to enhance the efficiency of surface colonization by *P. fluorescens*^{23, 27, 51, 53, 67} and *P. aeruginosa*⁶⁶. The dependence on flagella is conditional, however, as non-motile mutants of *P. fluorescens* and *P. aeruginosa* were deficient in biofilm formation in glucose minimal medium, but proficient in biofilm formation in citrate minimal medium^{47, 67}.

2.1.2. Attachment to the Substratum. It appears that the pseudomonads possess a range of different adhesins that can function in initial attachment to a substratum and biofilm formation. Type IV pili were shown to be important for attachment of *P. aeruginosa* to biotic surfaces⁷⁴, and hyperpiliated phase variants of *P. aeruginosa* rapidly initiated formation of strongly adherent biofilms on abiotic surfaces²⁸. The conditional dependence on flagella for surface colonization described above may, besides a role in bacterial transport to the surface, be because of adhesion properties of the flagella. *P. fluorescens* cells were shown to attach apically to a glass surface in flow-cells and rotate, indicating that the initial attachment occurred by means of flagella⁵⁴. The initial attachment was reversible, but cells became irreversibly attached by progressing from apical to longitudinal attachment. *P. aeruginosa* was found to display similar behaviour during colonization of a glass surface in flow-cells⁷⁹. Non-flagellated mutants of *P. fluorescens* and *P. aeruginosa* attached longitudinally^{53, 79}, indicating that flagella are not required for attachment *per se* but may enhance the process. Genes encoding chaperone usher pathways for synthesis of as yet uncharacterized fimbrial adhesins were shown to be necessary for initial *P. aeruginosa* biofilm development⁹⁰. In addition to type IV pili, flagella, and fimbria, outer membrane proteins may play a role in attachment. A *P. putida* mutant, which was originally

isolated because of a defect in adhesion to seeds, and was inactivated in a gene encoding a putative calcium binding outer membrane protein, was shown also to be deficient in biofilm formation on abiotic surfaces³². Moreover, extracellular DNA was shown to be an important constituent in the formative stages of *P. aeruginosa* biofilm formation⁹⁵, and a type IV hyperpiliated *P. aeruginosa* mutant inactivated in a gene, *pilU*, encoding a DNA binding protein^{1, 11}, was defective in biofilm formation under flowing conditions⁹.

2.1.3. Formation of Initial Microcolonies. The cellular adhesiveness mediated by the factors involved in surface attachment probably also play a role as cell-to-cell adhesins in the earliest phase of microcolony formation. The small microcolonies formed by *P. aeruginosa* in the initial phase of biofilm formation could be dissolved by exogenous DNase activity indicating a role for extracellular DNA as a cell-to-cell glue in the earliest phase of *P. aeruginosa* biofilm formation⁹⁵.

The initial biofilm development by *P. aeruginosa* appears to be conditionally dependent on type IV pili. With glucose as carbon source type IV pili mutants are deficient in biofilm formation under static conditions^{47, 66}, but proficient in biofilm formation under flowing conditions⁴⁶. With citrate as carbon source type IV pili mutants are proficient in biofilm formation both under static conditions and flowing conditions^{41, 47}. Mutants with lesions in genes that affect type IV pili expression, such as the global carbon metabolism regulator, *crc*, or the polyphosphate kinase gene, *ppk*, are also deficient in biofilm formation in glucose medium under static conditions^{64, 75}. The observation that type VI pili mutants were able to form a monolayer of attached cells but not microcolonies (cell clusters) in a static system with glucose as carbon source, led to the suggestion that microcolony formation during biofilm development by *P. aeruginosa* occur through type IV pili-driven aggregation⁶⁶. However, the use of colour-coded bacteria and time-lapse confocal laser scanning microscopy (CLSM) showed that formation of the initial microcolonies during biofilm development by *P. aeruginosa* and *P. putida* under flowing conditions occurs through clonal growth^{46, 47, 89}. In these systems the initial microcolonies are formed by cells that do not display twitching motility and therefore proliferate at fixed positions, while cells that display twitching motility move on the substratum and do not participate in formation of the initial microcolonies⁴⁶.

The global regulatory gene *GacA* has been shown to be involved in initial biofilm development by *P. aeruginosa*⁶⁹, indicating the existence of biofilm-specific gene expression.

2.2. Gene Expression During Initial Biofilm Development

Analysis of population-level average gene expression in biofilms has been done by reporter gene, proteome, or transcriptome analysis of cells

obtained from biofilms. Analysis of gene expression in individual cells situated in biofilms has been done by the use of *in situ* reporter genes that can be directly monitored at the single-cell level.

2.2.1. Global Analysis of Gene Expression During Initial Biofilm Development.

A global analysis of average physiological changes in *P. putida* populations following 6 hr of attachment to a silicone surface was carried out by the use of 2-D protein gel electrophoresis and cDNA subtractive hybridization⁷⁸. The proteome analysis revealed 45 differences in the protein profiles of planktonic and sessile cells, and the subtractive hybridization analysis revealed at least 40 differences in mRNAs of planktonic and sessile cells. Proteins involved in amino acid metabolism, an outer membrane lipoprotein, NlpD, a putative ABC transporter, PotF, and proteins involved in flagellar synthesis, were downregulated following 6 hr of attachment. Proteins involved in synthesis of type IV pili (PilR, PilC, and PilK) and lipopolysaccharides (LpxD and WbpG), and the ABC transporter PotB, were upregulated following 6 hr of attachment.

2.2.2. In Situ Monitoring of Gene Expression During Initial Biofilm Development.

Using an *algC::lacZ* fusion reporter gene in *P. aeruginosa*, and a fluorogenic β -galactosidase substrate suitable for single-cell *in situ* monitoring, it was shown that cells which had attached to a Teflon mesh or glass surface for 15 min exhibited up-expression of the alginate biosynthesis gene *algC*^{17, 18}. Initial cell attachment to the substratum appeared to be independent of *algC* promoter activity, but cells not exhibiting *algC* up-expression were shown to be less capable of remaining at a glass surface under flowing conditions than were cells in which *algC* up-expression was detected¹⁸, indicating that expression of exocellular polymeric substances may play a role in the early phase of biofilm development.

3. FURTHER BIOFILM DEVELOPMENT AND MATURATION

The formation of initial microcolonies, although it may occur through multiple pathways, appears to be a common trait in biofilm formation by the pseudomonads. Further biofilm development and maturation, however, is both species dependent, and dependent on the environmental conditions.

3.1. Structural Development

The use of confocal laser scanning microscopy (CLSM) to visualize fully hydrated living biofilms, pioneered by Lawrence *et al.*⁵⁵, has enabled very detailed studies of biofilm structural development. Biofilms had conventionally

been regarded as compact planar structures, but the first investigations with CLSM showed that biofilms are often open heterogeneous structures with interstitial voids separating discrete aggregates of cells tied together by extracellular polymeric substances^{22,55}. Statistical analysis by the use of COMSTAT image analysis of CLSM images have shown that biofilms formed by different pseudomonads often have unique architectures⁴². Since the structural development of *P. fluorescens* biofilms in many ways is similar to the structural development of *P. putida* biofilm, and since the structural development of *P. aeruginosa* biofilms differs substantially from that of *P. fluorescens* and *P. putida*, we will describe structural development of *Putida/P. fluorescens* biofilms and of *P. aeruginosa* biofilms separately below.

3.1.1. Structural Development in P. putida/P. fluorescens Biofilms. Studies using time-lapse CLSM of biofilms formed by colour-coded bacteria in flow-chambers irrigated with citrate minimal medium have provided knowledge about structural development in *P. putida* biofilms⁸⁹. Biofilm development occurs via formation of initial compact microcolonies by clonal growth of sessile cells at the substratum. When the microcolonies have reached a certain size (with citrate minimal medium, under flowing conditions at room temperature, this is after about 50 hr of biofilm development), the cells inside the microcolonies are no longer tied together and they exhibit excessive flagellum-driven motility. In a progression that lasts only a few hours, the biofilm dissolution process that initiated inside the microcolonies leads to dissolution of a substantial part of the microcolonies and to a shift in spatial organization from compact microcolonies to loose protruding structures⁸⁹ (see Figure 1). Further studies suggested that the local biofilm dissolution process is induced by carbon starvation, and that a signal for modulation of cell-to-cell interconnecting cellulase-degradable exopolymer is transduced by a GGDEF/EAL-domain containing regulatory protein³⁶.

P. putida biofilms grown on glutamic acid minimal medium under flowing conditions in silicone tubes appear to undergo a similar structural development⁷⁸. Immunoblot analysis suggested the absence of flagella on cells from 12-hr and 1-day-old biofilms, but the presence of flagella on cells from 3-day and 7-day-old biofilms⁷⁸, reflecting the role of flagellum-driven motility in biofilm development as described above.

Evidence that *P. fluorescens* undergo structural biofilm development similar to that displayed by *P. putida* has also been presented. Architectural analysis of *P. fluorescens* biofilms showed that the wild type, unlike a non-motile mutant, “developed zones near the base of the biofilm which possessed less cell material than was present in overlying cell layers,” and it was suggested that these “recurrent patterns present in biofilms likely represent an evolutionarily defined response by aerobic *P. fluorescens* biofilm-forming bacteria to oxygen- or nutrient-limiting growth conditions encountered within

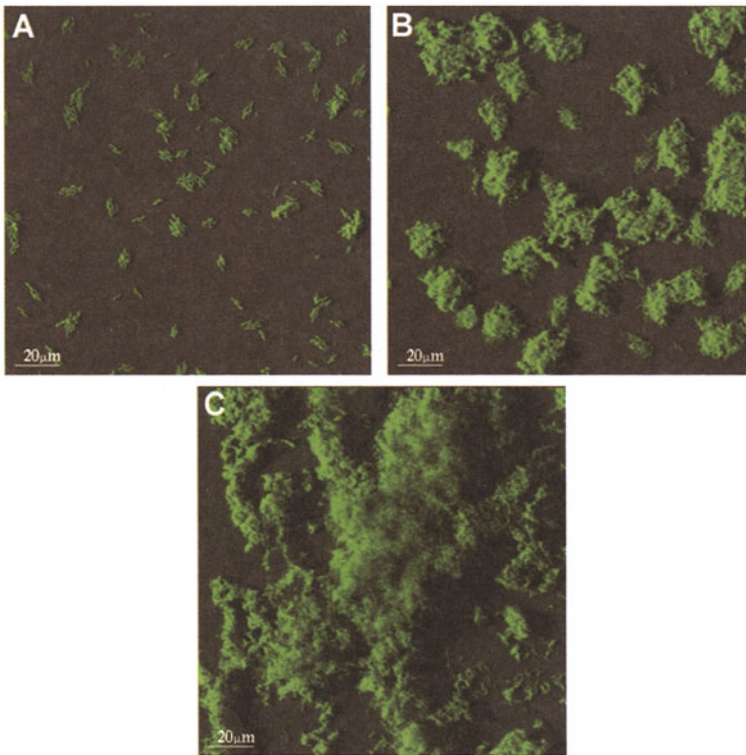


Figure 1. Structural development of a biofilm formed by green fluorescent protein-tagged *P. putida* in a flow-chamber irrigated with citrate minimal medium. CLSM shadows projection micrographs recorded in a (A) 1-, (B) 3-, and (C) 5-day-old biofilm are shown. Reproduced with permission from *J. Bacteriol.*, 182:6482–6489.

the film”^{50, 52}. As was found in the study of *P. putida* biofilms referred to above³⁶, bacterial cellulose-like exopolymer also appears to act as cell-to-cell interconnecting exocellular substance in *P. fluorescens* biofilms⁸².

3.1.2. Structural Development in *P. aeruginosa* Biofilms. Studies using time-lapse CLSM of biofilms formed by colour-coded bacteria in flow-chambers have provided knowledge about the structural development in *P. aeruginosa* biofilms^{46, 47}. The structural development of *P. aeruginosa* biofilms was shown to be dependent on the carbon source⁴⁷. When citrate was used as carbon source *P. aeruginosa* PAO1 formed a flat and very dynamic biofilm, whereas when glucose was used as carbon source *P. aeruginosa* PAO1 formed a heterogeneous biofilm containing mushroom-shaped multicellular structures separated by water-filled channels.

P. aeruginosa biofilm development in flow-chambers with citrate as carbon source occurs via formation of initial microcolonies by clonal growth of sessile cells at the substratum, whereafter the bacteria spread out on the substratum by type VI pili-driven twitching motility, in a process that, in combination with flagellum-driven emigration out of the biofilm, results in the formation of a flat biofilm⁴⁷. CLSM time-lapse microscopy of the biofilm development (see Figure 2) indicated that the shift from sessile to motile cells occurred when the initial microcolonies reached a certain size, suggesting that the shift was induced by some sort of nutrient limitation. Evidence has been presented that twitching motility may be stimulated by iron limitation⁸¹, and that it possibly may be directed by chemical gradients^{15, 44, 45}. In agreement with the structural development in citrate-grown *P. aeruginosa* biofilms, it was reported that a component of innate immunity inhibits the formation of large microcolonial structures in *P. aeruginosa* biofilms by stimulating twitching motility⁸¹.

The formation of mushroom-shaped structures in glucose-grown *P. aeruginosa* biofilms occurred in a sequential process involving a non-motile bacterial subpopulation which formed the initial microcolonies by growth in certain foci of the biofilm, and a migrating bacterial subpopulation which

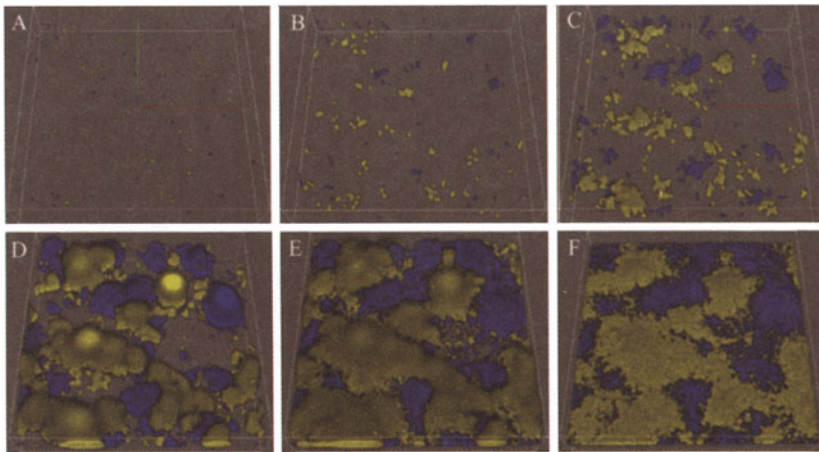


Figure 2. Time-lapse CLSM in a colour-coded *P. aeruginosa* PAO1 wild-type biofilm. The biofilm was initiated with a 1:1 mixture of yellow fluorescent protein-tagged and cyan fluorescent protein-tagged *P. aeruginosa* wild-type bacteria, and was grown in a flow-chamber on citrate minimal medium. The structural development in the biofilm was followed by time-lapse CLSM. The shown CLSM side view projection micrographs were acquired after (A) 0, (B) 7, (C) 11, (D) 15, (E) 19, and (F) 23 hr of biofilm development. Reproduced with permission from *Mol. Microbiol.*, 48:1511–1524.

initially formed a monolayer on the substratum, and subsequently formed the mushroom caps by climbing the microcolonies (mushroom stalks) and aggregating on the tops in a type-IV pili-driven process⁴⁶. Growth of the initial microcolonies in the glucose-grown biofilm continues past the point where spreading by twitching motility prevents further microcolony-formation in the citrate-grown biofilm. Formation of mushroom-shaped structures did occur in citrate-grown *pilA*: wild-type mixed biofilms, where the *pilA* bacteria functioned as the mushroom stalk-forming subpopulation (see Figure 3), suggesting that the difference in structure between the citrate-grown and the glucose-grown *P. aeruginosa* wild-type biofilms is caused mainly by different capabilities of forming the mushroom stalks⁴⁶. Microcolony formation may continue in certain foci of the glucose-grown *P. aeruginosa* biofilm as a consequence of twitching motility suppression in response to local environmental cues. However, it is also possible that stalk formation occurs because the cells in certain foci of the biofilms produce cell-to-cell interconnecting compounds and adhere strongly to each other so that twitching motility becomes arrested. Since chemotaxis-related genes are involved in twitching motility by *P. aeruginosa*¹⁵, and local consumption creates nutrient gradients in biofilms^{22, 71, 96}, it is possible that the migrating bacteria climb on top of the existing microcolonies because more nutrients are available on the top. Twitching motility is not regulated by any of the known cell-to-cell signalling systems in *P. aeruginosa*⁶, indicating that migration of the cells on top of the stalks is not coordinated by a known cell-to-cell communication system. Since a role of quorum-sensing in

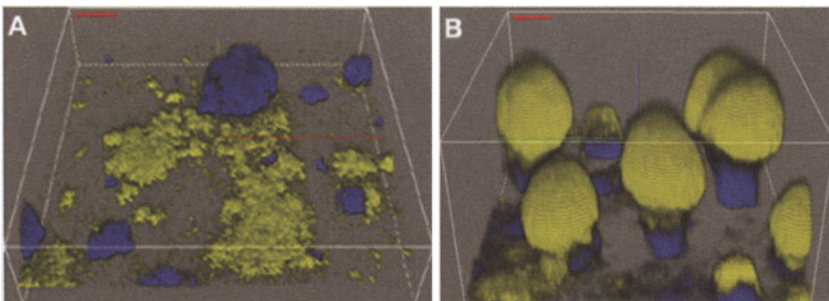


Figure 3. CLSM micrographs acquired in a (A) 1-day-old and (B) 4-day-old citrate-grown biofilm which was initiated with a 1:1 mixture of yellow fluorescent protein-tagged wild-type bacteria and cyan fluorescent protein-tagged *pilA* mutants. In flow-chambers irrigated with citrate minimal medium the *P. aeruginosa* wild-type bacteria (yellow) initially spread on the substratum by means of twitching motility, while the *P. aeruginosa pilA* bacteria (cyan) form microcolonies by clonal growth at fixed positions. Thereafter, the wild-type bacteria climb the *pilA* microcolonies and aggregate on the top, forming mushroom caps in a process which is dependent on type IV pili. Bars, 20 μm . Reproduced with permission from *Mol. Microbiol.* 50: 61–68.

formation of the heterogeneous *P. aeruginosa* biofilm structures has been shown¹⁸, there may, however, be parallel quorum-sensing controlled pathways required for mushroom cap formation. It has been shown that rhamnolipid surfactant production affects *P. aeruginosa* biofilm architecture, and that a *rhlA* mutant did not maintain open channels in biofilms¹⁶. Since rhamnolipid synthesis is quorum-sensing regulated⁷⁰, and surfactants may facilitate twitching motility, it is possible that quorum-sensing plays a role in mushroom cap formation through these processes. Quorum-sensing could also be involved in a process that makes the twitching bacteria settle on top of the mushrooms. Since the presence of the stalk-forming *P. aeruginosa pilA* bacteria also resulted in mushroom formation in a citrate-grown *pilA*: wild-type mixed biofilm, where the wild-type bacteria exhibit extensive twitching motility and unaccompanied form a flat biofilm, it is possible that formation of some kind of cell-to-cell glue causes the twitching bacteria to settle on top of the mushrooms.

The structural development in *P. aeruginosa* biofilms grown on glutamic acid under flowing conditions⁷⁹ appears to resemble the structural development in the glucose-grown biofilms described above. Although Sauer *et al.*⁷⁹ used phase contrast microscopy in their analysis, which does not allow three-dimensional imaging, it appears that the stage referred to by those authors as maturation-1 corresponds to the biofilm with the initial microcolonies (mushroom stalks) formed by the non-motile subpopulation, and the monolayer on the substratum between the microcolonies formed by the migrating subpopulation; and that the stage referred to as maturation-2 corresponds to the biofilms with mushroom structures and little surface coverage, since the migrating population that covered the substratum in the maturation-1 stage has climbed the mushroom stalks and forms the mushroom caps in the maturation-2 stage. The maturation-1 stage was shown to be accompanied by the activation of the Rhl quorum-sensing system activating among other genes expression of *rhlA* encoding a rhamnolipid synthetase⁷⁹.

Alginate overproduction has been shown to affect the structural development of *P. aeruginosa* biofilms as alginate-overproducing strains formed biofilms with microcolonies of increased size^{40, 62}. During the course of chronic cystic fibrosis lung infections *P. aeruginosa* undergoes a conversion to a mucoid phenotype, which is characterized by overproduction of alginate²⁹.

3.1.3. Structural Development in a *Pseudomonas*–*Burkholderia* Two-Species Biofilm. Evidence that chemotactic behaviour may play a role in biofilm structural development was provided from studies of a two-species consortium capable of carrying out commensal metabolism⁶¹. In this model system *Burkholderia* sp. LB400 was capable of degrading 3-chlorobiphenyl to 3-chlorobenzoate, while *Pseudomonas* sp. B13 could mineralize 3-chlorobenzoate, but could not degrade 3-chlorobiphenyl. When the biofilm was grown on

3-chlorobiphenyl medium it developed into mixed microcolonies which contained *Burkholderia* sp. LB400 and *Pseudomonas* sp. B13 in tight physical association. When the biofilm was grown on citrate medium (metabolizable by both organisms), it developed into separate microcolonies which contained either *Burkholderia* sp. LB400 or *Pseudomonas* sp. B13. When a citrate grown biofilm was shifted to growth on 3-chlorobiphenyl, the structure changed towards mixed microcolonies within 2 days after the substrate shift. Additional experiments indicated that *Pseudomonas* sp. B13 is chemotactic towards 3-chlorobenzoate (Tolker-Nielsen, unpublished), suggesting that the substrate-induced structure formation may be a consequence of chemotactic behaviour. The shift in biofilm structure, which occurred after the shift from citrate medium to 3-chlorobiphenyl medium, probably also included local carbon starvation-induced biofilm dissolution prior to the migration of the *Pseudomonas* sp. B13 bacteria.

3.2. Gene Expression During Further Biofilm Development and Maturation

As for the young biofilms, analysis of population-level average gene expression in mature biofilms has been done by reporter gene, proteome, or transcriptome analysis, and analysis of gene expression in individual cells situated in biofilms has been done by the use of *in situ* reporter genes.

3.2.1. Global Analysis of Gene Expression in Mature Biofilms. Average gene expression in *P. aeruginosa* cells from mature biofilms has been compared with gene expression in *P. aeruginosa* cells from planktonic chemostat culture by the use of transcriptome microarray analysis⁹⁴. Only 73 (i.e., about 1%) of the genes showed differential expression in the two growth modes. Thirty-four of the genes were activated and 39 of the genes were repressed in biofilm cells in comparison to planktonic cells. About one third of the 73 biofilm-regulated genes encoded hypothetical proteins of unknown function. The most highly activated genes were those of a temperate bacteriophage that is closely related to bacteriophage Pf1. Type IV pili and flagella were downregulated on average in the cells from these biofilms which were structurally heterogeneous (as opposed to flat). The TolA membrane protein and the OmlA membrane lipoprotein, both of which may play a role in antibiotic tolerance, were induced in the biofilm cells. The *rpoS* gene in the biofilm cells was found to be repressed by the microarray analysis⁹⁴, but to be induced in *P. aeruginosa* biofilm cells by reporter gene and immunoblot analysis⁹⁸. The *rpoS* gene is also highly induced in *P. aeruginosa* cells from the lungs of cystic fibrosis sufferers³⁴.

Gene expression in *P. aeruginosa* biofilm and planktonic cells has also been compared by the use of 2-D protein gel electrophoresis⁷⁹. When planktonic

cells were compared with cells from a mature biofilm (referred to as maturation-2 stage), over 50% of the proteome were shown to have a sixfold or greater change in expression level. The transcriptome study referred to above is expected to identify gene expression levels in the mature biofilm only, whereas the proteome study (which did not use pulse-chase protein labelling) is expected to identify proteins transiently synthesized during the transition from planktonic through various biofilm stages in addition to proteins synthesized in the mature biofilm.

Genes upregulated in mature *P. aeruginosa* biofilms have also been identified by the use of *in vivo* expression technology (IVET; see Chapter 11 in Volume 1)³³. Genes encoding a porin homologue (PA0240), a putative alcohol dehydrogenase (PA3710), and a homologue of the *Streptomyces* developmental regulator (PA3782) were identified.

The difference in gene expression between biofilm cells and planktonic cells has led to the suggestion that biofilm cells may be differentiated through a process of microbial development⁶⁵. However, since liquid close to surfaces has changed physical and chemical properties³⁷, and since the metabolic processes carried out by the cells in a structured biofilm creates gradients of various compounds (see below), the difference in gene expression could alternatively be because the cells in biofilms are situated in various micro-environments and regulate gene expression accordingly.

3.2.2. In Situ Monitoring of Gene Expression in Mature Biofilms. Compartmentalization of specific gene expression in biofilms has been observed in studies of a *P. putida*–*Acinetobacter* sp. C6 consortium^{10, 60}. The studies employed a *P. putida* reporter bacterium with a chromosomal insertion of a mini-Tn5 cassette containing the TOL *xylS* gene and a fusion between the Tol *Pm* promoter and the *gfp* gene. Since XylS in combination with benzoate activates expression from the *Pm* promoter⁷³, the reporter strain becomes green fluorescent in the presence of benzoate. When a mixed-species biofilm containing *Acinetobacter* sp. C6 and the *P. putida* reporter strain was grown on benzyl alcohol, the *P. putida* reporter bacteria in the vicinity of *Acinetobacter* sp. C6 microcolonies expressed the *Pm::gfp* reporter gene indicating that the *Pm* promoter in the monitor-bacteria was activated by benzoate excreted from the *Acinetobacter* sp. C6 bacteria. In addition, use of an *in situ* growth activity reporter (described in detail later) showed that the *P. putida* cells in the vicinity of *Acinetobacter* sp. C6 microcolonies were the most active (see Figure 4).

The metabolic interactions in the commensal *Pseudomonas*–*Burkholderia* model-consortium described previously was also investigated by *in situ* analysis using the *xylS*–*Pm*–*gfp* monitor cassette⁶¹. XylS also activates transcription from the *Pm* promoter in combination with 3-chlorobenzoate⁷³, and when a biofilm consisting of *Burkholderia* sp. LB400 and *Pseudomonas* sp.

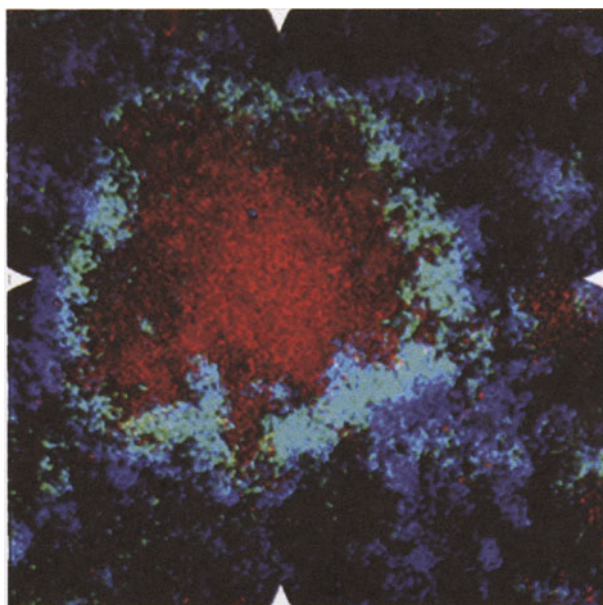


Figure 4. CLSM micrograph showing metabolic interaction between *P. putida* and *Acinetobacter* sp. C6 in a biofilm grown on benzylalcohol for 3 days. The biofilm was *in situ* rRNA hybridized with a *P. putida*-specific CY5-labelled probe (blue) and an *Acinetobacter*-specific CY3-labelled probe (red). The more active *P. putida* cells had increased Gfp content due to the expression of an *rrnBP1::gfp* (unstable) fusion, and appear cyan as the combination of green (GFP) and blue (hybridization).

B13::xylS-*Pm-gfp* grew on 3-chlorobiphenyl, the monitor-bacteria in the vicinity of *Burkholderia* sp. LB400 microcolonies were green fluorescent indicating that the *Pm* promoter in the monitor-bacteria was activated by 3-chlorobenzoate excreted from *Burkholderia* sp. LB400.

Quorum-sensing induced genes in *P. aeruginosa* have also been studied in biofilms by the use of *in situ* methods as described below.

3.2.3. Quorum-Sensing in Biofilms. Many bacteria are able to sense their population density and adjust gene expression accordingly through a process termed quorum-sensing. For a detailed description of the quorum-sensing process the reader is referred to Chapter 1 in Volume 2.

Quorum-sensing has been shown to have a role in the later stages of *P. aeruginosa* biofilm development¹⁹. A *P. aeruginosa lasI* mutant, deficient in the synthesis of the C₁₂ homoserine lactone quorum-sensing signal molecule, formed flat and sodium dodecyl sulphate-sensitive biofilms, as opposed to the

wild-type which formed heterogeneous biofilms with mushroom-shaped structures which were sodium dodecyl sulphate resistant. A role of quorum-sensing in the formation of the heterogeneous *P. aeruginosa* biofilms is consistent with the finding that a *P. aeruginosa* wild-type and *lasI* mutant formed similar flat biofilms in citrate medium⁴¹, as citrate medium, unlike glucose medium, does not support formation of the heterogeneous *P. aeruginosa* biofilm⁴⁷.

Temporal and spatial studies of the expression of *lasI* and *rhII* in *P. aeruginosa* biofilms was done by the use of fusions of the promoters to unstable versions of *gfp*²⁴. Unstable *gfp* versions allowing monitoring of real-time gene expression had been constructed by introducing a peptide tail rendering the *gfp* variant target for a tail-specific protease³. During the course of 8-day biofilm development, *lasI* expression was found to progressively decrease over time, whereas *rhII* expression remained steady throughout biofilm development but occurred in a lower percentage of the cells²⁴. The *lasI* and *rhII* genes were maximally expressed in cells located at the substratum and expression decreased with increasing biofilm height.

A similar reporter system for *in situ* monitoring of quorum-sensing in *P. aeruginosa* biofilms used fusions between the unstable *gfp* variant and the *lasB* promoter³⁹. It was shown that a synthetic halogenated furanone compound, which is a derivative of secondary metabolites produced by the macroalga *Delisea pulchra*, is capable of inhibiting quorum-sensing in *P. aeruginosa* biofilms. The furanone compound did not affect initial attachment to the substratum, but it did affect the architecture of the biofilm and enhanced the process of bacterial detachment, leading to a loss of bacterial biomass from the biofilm. Another study used this *in situ* reporter system to demonstrate homoserine lactone mediated communication between *P. aeruginosa* and *Burkholderia cepacia* in mixed-species biofilms⁷⁶.

Quorum-sensing also appears to play a role in biofilm formation by *P. putida* IsoF⁸⁴. The wild type formed homogenous unstructured biofilms, whereas a quorum-sensing mutant formed structured biofilms with micro-colonies and water filled channels. Since the quorum-sensing regulon in this *P. putida* strain includes a long-chain fatty acid CoA ligase, the difference in biofilm structures between the quorum-sensing mutant and the wild type was suggested to be caused by differences in cell surface properties, but a comparison of the whole-cell fatty acid profiles of the wild type and the quorum-sensing mutant did not reveal any significant differences⁸⁴.

The specific quorum-sensing regulated genes that are directly involved in biofilm structural development and biocide resistance remain to be identified. The studies of the role of quorum-sensing in *P. aeruginosa* and *P. putida* biofilms referred to above indicate that the effect of quorum-sensing on biofilm structure is conditional and differs among species. Since microarray analysis of the *P. aeruginosa* quorum-sensing regulon suggested that

conservatively 616 genes may be classified as quorum-sensing regulated⁹¹, the possibility exists that mutations in the quorum-sensing control system will have pleiotropic effects with complex consequences for many gene activities, which could affect biofilm development. These effects could be different for biofilms of the same strain grown under different sets of conditions, and would most likely be different in different species.

4. BIOFILM DISSOLUTION AND DISPERSAL

In addition to the mechanisms involved in biofilm formation, biofilm bacteria also possess mechanisms to change their adhesiveness and to break down or modulate the biofilm matrix. Emigration of cells from biofilm communities may be necessary to spawn novel communities at new locations, and migration of cells may allow sessile communities to change spatial organization in response to changing environments (e.g., as described in the *Pseudomonas* sp. B13–*Burkholderia* sp. LB400 mixed-species biofilm above). Detachment from biofilms of *Pseudomonas* sp. S9 in response to starvation, and of *P. fluorescens* in response to glucose, nitrogen, or oxygen depletion, has been reported^{4, 26, 97}. The involvement in biofilms of outer membrane components and exopolymers that hold the microbes together in distinct spatial structures, implies that dissolution or modulation of the interconnecting components is of importance when emigration or structural rearrangements are needed. In accordance, polysaccharide lyases have been proposed to have a role in biofilm dissolution for *P. aeruginosa*, *P. fluorescens*, and *Pseudomonas syringae*^{4, 7, 8, 68}.

Rapid dissolution of *P. putida* OUS82 biofilms was shown to occur in response to carbon starvation³⁶. A *P. putida* mutant deficient in biofilm dissolution was isolated and found to be inactivated in the gene PP0164. The PP0164 gene encodes a putative periplasmic protein of unknown function, and is located next to the gene PP0165 which encodes a putative transmembrane protein with a GGDEF domain. Proteins with the GGDEF domain are involved in the regulation of cellular adhesiveness in a wide range of bacterial species, many of which use bacterial cellulose as cell-to-cell interconnecting adhesin^{5, 77, 82, 88, 100}. A mutant inactivated in the PP0165 gene was found to be deficient in biofilm formation, and a plasmid-encoded GGDEF-containing protein was shown to complement the PP0165 mutation, and to activate the production of cellulase-degradable exopolymer in *P. putida* (Tolker-Nielsen and Molin, unpublished). In agreement with a role of cellulase-degradable exopolymer as cell-to-cell interconnecting compound in *P. putida* biofilms, treatment of the biofilms with cellulase resulted in biofilm dissolution³⁶. It is possible that the PP0164 gene product regulates biofilm dissolution by modulating the activity of the PP0165 gene product which regulates production of cellulase-degradable exopolymer.

A mechanism that involves phage-mediated cell-killing initiating inside microcolonies was proposed to facilitate dispersal of cells from mature *P. aeruginosa* biofilms⁹³. A subpopulation of viable cells that could disperse from the biofilm was always observed inside the regions affected by the phage. The Pfl-type filamentous phage implicated in cell-killing exists as a prophage in the genome of *P. aeruginosa*, and has been shown by transcriptome⁹⁴ and proteome⁷⁹ analysis to become induced in mature biofilms.

5. DIFFUSION, GRADIENTS, AND PHYSIOLOGICAL HETEROGENEITY IN BIOFILMS

Studies of liquid flow and molecular diffusion in heterogeneous biofilms have led to the proposal that the channels and interstitial voids separating microcolonies may function as a circulation system for efficient nutrient supply and waste product removal¹³.

Local fluid velocity in a mixed-species biofilm consisting of *P. aeruginosa*, *P. fluorescens*, and *Klebsiella pneumoniae* has been measured by the use of particle image velocimetry and CLSM^{20, 87}. Fluorescent beads were observed to flow through channels and accelerate when they passed through channel constrictions. Velocity profiling showed that the velocity of the flow in the biofilm could be related to physical parameters such as local structure and cell-cluster to void ratio, and that the path of liquid flow in the channels was directed by the shape of the channels.

Measurements of local diffusion by micro-injection and CLSM in the *P. aeruginosa*, *P. fluorescens*, and *K. pneumoniae* mixed-species biofilm showed that the diffusion coefficients of fluorescein in cell clusters and voids were similar, suggesting that the diffusivities of nonbinding compounds with molecular weights comparable to that of fluorescein (MW 332), such as many biocides and antibiotics, are not significantly decreased in the microcolonies of biofilms²¹. The diffusion of larger molecules such as phycoerythrin (MW 240,000) and IgG (MW 150,000) was hindered in the microcolonies, but IgG diffusion was influenced much more by binding to bacterial cells and matrix polymers than by physical obstruction, and the diffusivity was expected to be higher if the binding sites had been saturated. Monitoring of the mobility of fluorescently labelled dextrans in *P. fluorescens* and mixed-species biofilms by the use of CLSM and fluorescence recovery after photobleaching (FRAP) also showed that the diffusion of large molecules was hindered in the microcolonies in comparison to the bulk liquid⁵⁶. Regional variability in the mobility of the dextrans occurred in the mixed-species biofilms, with some regions exhibiting rapid diffusion of all test molecules, and adjacent regions penetrated only by lower-molecular-weight compounds.

Oxygen distribution and mass transfer was measured in biofilms by the use of microelectrodes and CLSM²². The oxygen distribution in the biofilm was strongly correlated with the structure of the biofilm. The voids between the microcolonies facilitated oxygen transport from the bulk liquid through the biofilm, supplying about half of the total oxygen consumed by the cells. Although oxygen was supplied to the microcolonies through all microcolony–liquid interfaces, the largest oxygen gradients existed in microcolonies facing bulk liquid or vertical voids, while smaller oxygen gradients were found in microcolonies facing voids lying in the horizontal direction below the biofilm surface.

Using CLSM and *in situ* hybridization with a fluorescent probe targeting *P. putida* and another probe targeting all eubacteria, the 3-D structure of a toluene-fed multi-species biofilm, and the activity of a toluene degrading *P. putida* biofilm member, were investigated⁵⁹. Microcolonies of the *P. putida* strain were found throughout the heterogeneous channel-containing biofilm, and judged from the ribosomal RNA content of the hybridized *P. putida* cells, they were equally active independent of their location, indicating that substrate was supplied via the channels to the inner regions of the biofilm.

Although the channels in biofilms may facilitate nutrient and oxygen transport by convection, and molecules with the size of nutrients and oxygen may easily diffuse into the biofilm microcolonies, there are many examples that due to consumption by the cells the penetration of nutrients and oxygen in biofilms is limited, and growth in many biofilms consequently occurs mostly in the outer layers of the microcolonies. In the study of oxygen distribution in biofilms referred to above, oxygen was found to penetrate only 30 μm into the microcolonies leaving the inner of the microcolonies anaerobic²². Another study used alkaline phosphatase (APase) as a reporter of physiological activity⁴³. Induction of APase occurs after phosphate deprivation preferentially in actively growing cells, and when *P. aeruginosa* biofilms were shifted to low-phosphate medium, APase activity was detected (using a fluorogenic substrate) in a well-defined band immediately adjacent to the biofilm–bulk fluid interface⁴³. Evidence was subsequently presented that growth in the interior of the *P. aeruginosa* biofilms was oxygen limited⁹⁹. Observations of increased growth activity of biofilm cells closest to the channels and bulk liquid have also been made from studies with gyrase inhibiting compounds (such as nalidixic acid and fleroxacin), which can be used to estimate cell growth potential on the basis of the amount of cell elongation that occurs after exposure. Fleroxacin treatment of a *P. fluorescens* biofilm caused a gradient of cell elongation, with the greatest amount of cell elongation occurring near the microcolony–liquid interfaces⁵⁰. Physiological heterogeneity in biofilms has also been investigated using *in situ* growth activity reporter genes⁸⁵. A fusion between the *Escherichia coli* ribosomal *rrnBP1* promoter and an unstable Gfp

protein was constructed and inserted into the chromosome of *P. putida* resulting in a growth activity reporter strain which emits green fluorescence with an intensity proportional to the growth activity. When biofilm microcolonies of this *P. putida* strain reached a critical size, the light emitted by the cells decreased in the centre of the microcolonies and eventually throughout the microcolonies, showing that the cells displayed different levels of growth activity correlating with their location in the biofilm and with the biomass of the biofilm.

6. BIOFILM RESISTANCE

Because of their innate resistance to host immune systems, antibiotics, and biocides, biofilms in medical and industrial settings are difficult to eradicate, and biofilm formation therefore leads to various persistent and sometimes lethal infections in humans and animals, and to a variety of problems in industry where solid–water interfaces occur^{12, 14}. It has been estimated that biofilms are the causative agent of up to 65 percent of bacterial infections⁷².

Although molecules with the size of biocides and antibiotics readily diffuse in biofilms²¹, in some cases where the biocides or antibiotics bind to the cells or to extracellular substances, biofilm resistance may be caused by poor penetration of the antibiotics into the deeper layers⁸⁶. Hence biofilms formed by an alginate-overproducing *P. aeruginosa* strain were more resistant to tobramycin than biofilms formed by the wild type⁴⁰. As tobramycin and ciprofloxacin were shown to penetrate *P. aeruginosa* wild-type biofilms but at the same time fail to effectively kill the bacteria⁹², it appears that limited antibiotic diffusion may not to be the primary protective mechanism for biofilms.

Since slow growing or stationary phase cells may be resistant to antibiotics^{31, 35}, the physiological heterogeneity of biofilms (described above) may be a cause of resistance. Evidence was provided that biofilms and stationary phase planktonic cells of *P. aeruginosa* have similar resistance to killing by antibiotics, and that resistance to those antibiotics that may kill stationary phase cells is dependent on a subpopulation of persister cells⁸³. By the use of microelectrodes and microscopic analysis in a biofilm containing a *P. aeruginosa in situ* growth activity reporter strain, it was shown that oxygen limitation and low metabolic activity in the interior of the biofilm correlated with antibiotic tolerance⁹².

It has been suggested that bacteria growing in biofilms undergo distinct phenotypic changes that render them more resistant to biocides and antibiotics than planktonic cells¹⁴. As described previously, studies of global gene expression

in biofilms have provided limited information about the nature of such phenotypic changes. It has been shown, however, that antibiotic-resistant phenotypic variants of *P. aeruginosa* with enhanced ability to form biofilms arise at high frequency both *in vitro* and in the lungs of cystic fibrosis patients³⁰. In addition, there is some evidence that quorum-sensing in biofilms may govern specific gene expression to modulate resistance to antibiotics. While biofilms formed by *P. aeruginosa* wild-type bacteria were highly resistant to kanamycin, biofilms formed by a *lasI* quorum-sensing mutant were susceptible to kanamycin, and biofilms formed by a *rhII* quorum-sensing mutant showed reduced resistance to kanamycin⁸⁰. Quorum-sensing also plays a role in the resistance of *P. aeruginosa* biofilms to sodium dodecyl sulphate (as described previously) and hydrogen peroxide^{19, 38}. Because multidrug resistance efflux pumps play an important role in the resistance of planktonic *P. aeruginosa* cells to antibiotics^{2, 48, 49, 57, 63}, their expression patterns and contributions to antibiotic resistance in *P. aeruginosa* biofilms were investigated²⁵. The analysis showed that the four characterized efflux pumps in *P. aeruginosa* do not play a role in the resistance of *P. aeruginosa* biofilms to antibiotics.

7. CONCLUSIONS

A substantial part of our knowledge about microbial biofilms has come from studies involving pseudomonads as model organisms.

The studies have shown that there is not one single biofilm developmental pathway. Instead, biofilm formation is conditional and may occur through multiple pathways. Different mechanism for initial attachment and microcolony formation may be used under different conditions, and structural development and maturation may occur differently under different conditions. The fact that bacteria have many pathways for biofilm formation may indicate that various environmental niches are colonized by the bacteria through biofilm formation via different pathways, and that there is a strong selection for the ability to form biofilms in most niches.

Planktonic cells and biofilm cells differ with respect to gene expression. We still do not know whether these differences in gene expression are because biofilm cells may be differentiated through a process of microbial development, or because the cells in a biofilm are situated in various microenvironments and regulate gene expression accordingly. So far it has not been possible to discriminate the expression of a core of truly biofilm developmental genes from the expression of genes as a response to the heterogeneous biofilm environment. *In situ* analysis of the temporal and spatial expression in biofilms of interesting genes identified through global analysis may help resolve this issue.

Quorum-sensing plays a role in the formation of the heterogeneous *P. aeruginosa* biofilm, but does not play a role in the formation of the flat *P. aeruginosa* biofilm. Quorum-sensing deficient *P. aeruginosa* mutants form a flat biofilm under conditions where wild-type bacteria form a heterogeneous biofilm. Conversely, quorum-sensing deficient *P. putida* mutants form a heterogeneous biofilm under conditions where the wild-type bacteria form a flat biofilm. The specific quorum-sensing regulated genes that are directly involved in biofilm structural development and resistance remain to be identified. Since the *P. aeruginosa* quorum-sensing regulon comprises several hundred genes, the possibility exists that mutations in the quorum-sensing control system has pleiotropic effects with complex consequences for many gene activities, which could affect biofilm development. These effects would most likely be different in different species, and different for biofilms of the same species grown under different conditions.

The heterogeneous biofilm contains tower- or mushroom-shaped microcolonies separated by interstitial voids and channels. The heterogeneous biofilm structure evidently creates a circulation system which enhances nutrient supply and waste product removal. It is still an open question, however, whether the heterogeneous biofilm structure is created through a morphogenetic process¹³, or alternatively arises mainly due to limitations in nutrient or oxygen transfer^{71, 96}. It seems clear, though, that multicellular structures in biofilms can arise both due to growth differentiation and due to bacterial migration.

The increased resistance of biofilms towards antibiotics and biocides may be caused by many factors. In some cases it appears that biofilm cells acquire increased resistance properties via a process that involves quorum-sensing. In cases where the antimicrobial compound binds to cells or exocellular substances, poor penetration may protect the cells positioned inside microcolonies. The presence of stationary phase cells, phenotypic variants, and persister cells in the physiological heterogeneous biofilms may also cause biofilm resistance.

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CELL ARCHITECTURE

THE OUTER MEMBRANES OF PSEUDOMONADS

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1. INTRODUCTION

The study of bacterial cell surfaces began in 1675 when Leeuwenhoek peering through his microscope wondered what “held [bacteria] together, or what contained them”⁹². Since that time, our knowledge of cell surfaces, namely the Gram-negative outer membrane, has grown considerably. In addition to containing the bacteria, the outer membrane mediates a myriad of functions including other structural roles such as maintaining bacterial shape and providing a scaffold for fimbriae and flagella. Another key function of the outer membrane is to mediate the interactions between Gram-negative bacteria and their environment, primarily by determining which compounds enter and exit the cell. This complex task involves the integration of lipidic components involved in the barrier function of the outer membrane and proteins involved in the uptake and efflux of the various compounds able to traverse this barrier.

Pseudomonads are intrinsically resistant to a large variety of toxic compounds including antibiotics, organic solvents, dyes, detergents, and heavy metals⁴⁰. Yet, at the same time, these organisms are able to take up an astonishing array of metabolites. Therefore the *Pseudomonas* outer membrane has emerged as a prototype to study the intricacies of this dynamic organelle. This chapter covers the structural basis of *Pseudomonas* outer membrane impermeability. The current status of *Pseudomonas* porins (upto April 2003) involved in

uptake is also discussed. We recently reviewed the structure, function, and genomics of the known and predicted *Pseudomonas aeruginosa* outer membrane proteins and refer the reader to this review for additional information⁴².

2. STRUCTURE OF THE OUTER MEMBRANE

The outer membrane is an atypical bilayer (Figure 1). The inner leaflet is composed of phospholipids similar in composition to those of the cytoplasmic membrane while the outer leaflet contains few, if any, of these same phospholipids, but is primarily composed of the complex glycolipid lipopolysaccharide (LPS). Embedded in this bilayer are a series of around 160 transmembrane

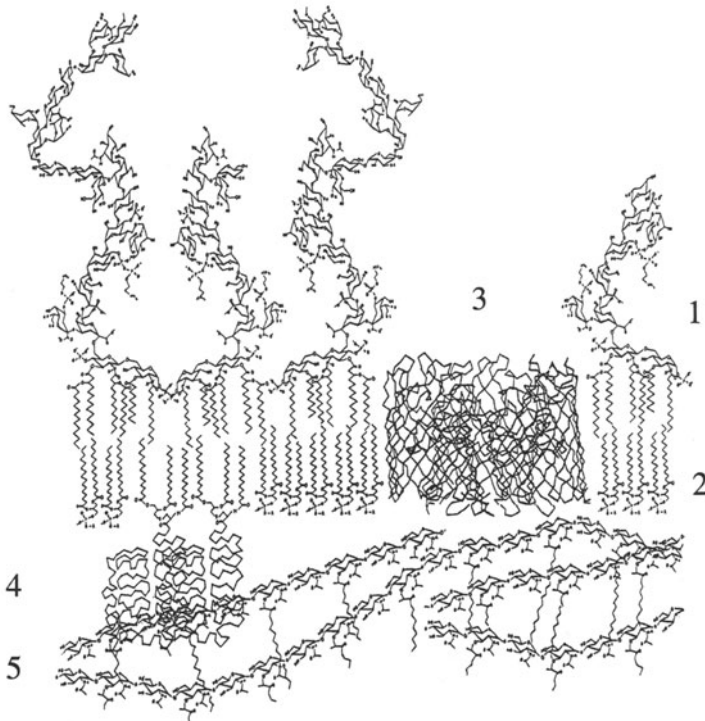


Figure 1. Representation of a typical *Pseudomonas* outer membrane. LPS molecules (1) comprise the majority of the outer leaflet. The inner leaflet is composed of phospholipids (2), mainly phosphatidylethanolamine. The outer membrane also contains proteins involved in transport, adhesion, membrane stability and recognition by the immune system. These proteins include porins (3) and lipoproteins (4). The lipoproteins anchor the outer membrane to the peptidoglycan (5) in the periplasm. This figure was originally published in ref. [43].

proteins, most of which have a β -barrel structure, and a few lipoproteins that are associated with one (usually the inner) monolayer. These components combine to confer on outer membranes their important properties that include the formation of a selective permeability barrier; participation in the relatively non-specific efflux, and specific export of molecules and proteins from cells; interaction with the immune system, immune cells, molecules and surfaces in the environment; an ability to exclude and resist enzyme attack; a structural role in cells; and the anchoring of structures like pili and flagella. We discuss below the specifics of the structure and functions of a subset of these outer membrane components. For a detailed description of the structure of outer membranes, we refer the reader to ref. [43].

One function of interest that is based on these components is antibiotic uptake. Some antibiotics pass across the outer membrane through the water-filled channels of porins (see below). In addition Gram-negative bacteria permit the passage of polycations across the outer membrane, using a system termed self-promoted uptake³⁸ that was first proposed based on studies of gentamicin and polymyxin B resistance in *P. aeruginosa* in 1981. In this hypothesis, polycationic compounds (i.e., those with two or more positive charges) such as trisaccharide aminoglycosides (the second most utilized antibiotics in hospitals), lipopeptide polymyxins (used intensively as topical agents), and cationic antimicrobial peptides, interact with bacterial cell surfaces at divalent cation binding sites on polyanionic LPS. These sites are normally involved in cross-bridging adjacent LPS molecules and stabilizing the outer membrane, but have 2–4 orders of magnitude higher affinity for the polycationic antimicrobials than for the native divalent cations (usually Mg^{2+} or Ca^{2+}). Since the polycationic antimicrobials are far bulkier than the divalent cations they competitively displace, they lead to a disruption of the outer membrane through which probe molecules, and more importantly the disrupting polycations themselves, traverse the outer membrane. Thus the name “self-promoted uptake” stems from the concept that these polycationic antibiotics are promoting their own uptake (e.g., as opposed to diffusing through the channels of porins).

2.1. Phospholipids

The phospholipids in the inner leaflet of the outer membrane are similar in composition to those of Gram-negative bacterial cytoplasmic membranes, consisting primarily of phosphatidylethanolamine, with smaller amounts of phosphatidylglycerol, diphosphatidylglycerol (cardiolipin), and other acidic phospholipids⁸⁴. Some *Pseudomonas* species may also have ornithine amine lipids in their outer membranes. These lipids are analogous to phospholipids and in *Pseudomonas fluorescens*, are generally synthesized in phosphate limiting conditions^{68, 96}.

The fatty acyl tails of the phospholipids are predominantly palmitate (C16:0), with lesser amounts of oleate (C18:1), and minor amounts of palmitoleate (C16:1). An important difference between the Pseudomonads and other Gram-negative organisms is that the predominant unsaturated fatty acid in Pseudomonads is C18:1 instead of C16:1⁷⁵. The predominance of a longer fatty acid could result in Pseudomonad outer membranes being more rigid than those of their Gram-negative counterparts.

In addition to the fatty acids mentioned above, *Pseudomonas* sp. produce an assortment of other fatty acids⁸⁴. The saturated species range from C10:0 to C17:0 and may be terminally branched. The unsaturated fatty acids range from 15 to 18 carbon atoms. These fatty acids are usually monounsaturated with *cis*-double bonds being predominant. As well, there are some species that produce cyclopropane fatty acids (either C17:Δ or C19:Δ). If isolates are grown in controlled conditions, this diversity in fatty acid profiles between species can be used to differentiate between them.

2.1.1. Changes in Fatty Acid Profile. Bacteria can alter their fatty acid profiles to make their membranes more resistant to environmental stresses such as exposure to organic solvents, temperature, heavy metals, and nutrient limitation⁸⁷. There are two means for achieving this result. The first is a short-term response involving the modification of the *cis*-unsaturated fatty acids into their *trans*-isomers by a periplasmic *cis-trans* isomerase. The second is a longer term response involving the differential synthesis of saturated fatty acids to increase their proportion in the membrane. Both mechanisms result in elongated fatty acid tails, which pack tightly leading to a more rigid membrane able to withstand harsher conditions while still maintaining transport activity.

2.2. LPS

The biosynthesis of Pseudomonad LPS will be described in detail in Chapter 1 of Volume 3 of this series. *P. aeruginosa* has two varieties of LPS molecules, the more-typical O-antigenic LPS, also called B-band LPS, and a common LPS referred to as A-band LPS (discussed later). LPS is a complex glycolipid with a tripartite structure. The endotoxic Lipid A moiety is inserted into, and is the major component of, the outer (surface) monolayer of the outer membrane. The structure of Lipid A is highly conserved among the Pseudomonads, consisting of a di-phosphorylated diglucosamine inserted into the membrane via several attached fatty acids. The core oligosaccharide is covalently attached to the Lipid A. Within a given species this region is similar in composition, and in addition to containing various sugars, in *Pseudomonas* harbours several phosphate molecules that together with anionic sugars and the Lipid A phosphates are the divalent cation binding sites of LPS.

The O-polysaccharide caps off about 10% of LPS molecules⁴³. This component, comprising a repeated tri- to tetra-saccharide is exposed to the external environment and is the most variable one, giving each *Pseudomonas* serotype its unique immunogenic properties⁷⁵.

2.2.1. Lipid A. The inner portion of LPS is Lipid A. In *Pseudomonas*, this moiety consists of a phosphorylated β -1,6-glucosamine disaccharide that is usually substituted with phosphate monoesters at the C1 and C4' positions. The disaccharide is anchored to the outer membrane by 6 or 7 fatty acyl chains linked through either ester or amide linkages⁷⁵. These fatty acids are predominantly hydroxyl fatty acids, although there may be small amounts of saturated fatty acids as well. The major hydroxyl fatty acyl chains in *Pseudomonas* are 3-hydroxydodecanoate (C3OH-12:0) followed by 2-hydroxydodecanoate (C2OH-10:0) and trace amounts of 3-hydroxydecanoate (C3OH-10:0); this is in contrast to the Enterobacteriaceae where 3-hydroxytetradecanoate (C3OH-14:0) predominates. The shorter hydroxyl fatty acyl chains in *Pseudomonas* may serve to make their outer membranes more fluid than those of their Gram-negative counterparts. Additionally, the hydroxyl group of these fatty acyl chains may be substituted with either a palmitate (C16:0) or another 2-hydroxydodecanoate (C2OH-12:0)⁴³. These substituents may influence the packing of the fatty acids and thus contribute to the fluidity of this region of the outer membrane, and its ability to intermediate in self-promoted uptake.

In addition to the major fatty acids mentioned above, the LPS molecules from different *Pseudomonas* species can contain minor hydroxyl fatty acyl chains. These fatty acyl chains range from 10 to 18 carbon atoms in length and may be saturated or monounsaturated, although saturated forms predominate⁸⁴. Terminally branched hydroxyl fatty acids have only been detected in one species, *Pseudomonas rubescens*, to date. As with the unsaturated fatty acyl chains, the diversity of hydroxyl fatty acyl chains can be used to classify *Pseudomonas* species. However, as fatty acyl profiles can change under different environmental conditions, the isolates must be grown under carefully controlled conditions.

2.2.2. Biological Role of Lipid A. Lipid A has an ability to induce the mammalian innate immune system by interacting with Toll-like receptor 4 on the surface of immune cells. Included in this is a pro-inflammatory response involving among other things the up-regulation, largely through transcription factor NF κ B, of the production of specific cytokines. If too large an LPS stimulus occurs (e.g., when *Pseudomonas* is treated with antibiotics causing it to release large amounts of LPS) a septic response, also called endotoxaemia, occurs and can lead to reduced blood pressure, organ failure, and death from endotoxic shock. Studies with synthetic Lipid A molecules indicate that the disaccharide backbone with phosphates placed at the C1 and C4' positions is

required for activation. The number and length of the acyl chains also influence the extent of the response. One of the most potent Lipid A moieties examined to date is that of *Escherichia coli*, which has 6 acyl chains that are 14 carbon atoms in length²⁸. The shorter hydroxyl fatty acid tails of the *Pseudomonads*' Lipid A moiety⁵² are probably responsible for the moderately decreased toxicity of their Lipid A cf. enterobacterial Lipid A⁸³. Other structural features of LPS, such as the core region, can also modulate the effect of Lipid A on the immune response⁵⁹.

The structure of Lipid A can change in response to environmental conditions. In *P. aeruginosa*⁶⁵ as with *Salmonella enterica* serovar Typhimurium³⁷, the PhoP–PhoQ two component regulatory system acts in response to low divalent cation (Mg^{2+} , Ca^{2+}) concentrations to promote *in vivo* survival of the bacteria. Under these conditions, in *Salmonella*, an operon encoding enzymes involved in adding 4-amino-4-deoxyarabinose residues to the Lipid A disaccharide is activated¹⁰³. This modification renders the bacteria more resistant to cationic antimicrobial peptides, presumably by decreasing the net negative charge of LPS. *P. aeruginosa* LPS isolated from the lungs of cystic fibrosis patients or from strains grown under low Mg^{2+} conditions is also modified with 4-amino-4-deoxyarabinose²⁷. It is believed that the mechanism for this alteration is related to that of *Salmonella* as *P. aeruginosa* possesses a similar LPS modification operon (PA3552–3558, ranging from 61% to 81% similar to the equivalent genes in *S. typhimurium*) that is activated in response to cationic antimicrobial peptides (J.B. McPhee and R.E.W. Hancock, manuscript submitted).

2.2.3. Core Oligosaccharide. Attached to the Lipid A is the core oligosaccharide. The core components are common to all *Pseudomonas* species examined to date and consist of sugar molecules including galactosamine, rhamnose, glucose, L-glycero-D-manno-heptose and the unique octose 2-keto-3-deoxyoctulosonic acid (KDO). The core is also differentially substituted with alanine and phosphate molecules. The *Pseudomonas* core region is rich in phosphate molecules, containing approximately twice the amount found in the Enterobacteriaceae⁷⁵. It has been suggested on immunological grounds that there are four core structures in *P. aeruginosa* but this may represent differential substitution or connectivity of the component sugars^{82, 111}. A similar trend is seen in the Enterobacteriaceae⁴⁶.

The phosphate molecules in the core region contribute to the barrier function of the outer membrane. The phosphorylated LPS imparts a strong net negative surface charge to the bacterium, which is partly neutralized by Mg^{2+} ions. This causes repulsion of strongly negatively charged substrates but permits the uptake of polycationic substances through the self-promoted uptake system. These sites are also presumably the sites for uptake of hydrophobic molecules, which can then be effluxed out of cells as described below.

2.2.4. O-polysaccharide. Capping off about 10% of LPS molecules and facing the external environment is the O-polysaccharide. This is the most diverse region of the LPS molecule and varies with respect to sugar composition, linkage, sequence, and branch length. A typical *Pseudomonas* O-polysaccharide chain is made up of repeating units of 3–5 sugars consisting of such sugars as glucosamine, glucose, rhamnose, fucosamine, and often amino hexuronic acids such as quinavosamine, 2-imidazolinomannuronic acid, and 2,3-diacetamido-2,3-dideoxyhexuronic acid⁶⁶.

P. aeruginosa is unusual among bacteria because most strains possess 2 forms of LPS, referred to as A-band and B-band LPS⁶⁰. The B-band confers serotype specificity to each strain. The A-band is common to all strains (and has also been observed in other *Pseudomonads*) and is composed mainly of trisaccharide repeats of D-rhamnose, with minor amounts of 3-O-methyl rhamnose, glucose, ribose, and mannose. The chains of A-band LPS are considerably shorter than those of B-band LPS. Studies with monoclonal antibodies to the core region of B-band LPS suggest that these two species of LPS share a common core⁹⁰. However, A-band chains contain sulphate instead of phosphate and have low levels of KDO and amino hexuronates. It is not clear whether there is a common Lipid A molecule for both LPS types.

The majority of LPS molecules on the cell surface are rough, in that the core is not capped with O-polysaccharide. Only 10% of the LPS molecules on a cell have an O-polysaccharide⁴³. These smooth LPS molecules are of variable length and result in a characteristic ladder pattern when resolved on an SDS-PAGE gel³⁹.

The term smooth LPS is derived from smooth appearance that the O-polysaccharide confers on bacterial colonies. The LPS forms a capsule-like covering that can extend up to 40 nm from the cell surface into the environment⁴³. Because of this exposure, the O-polysaccharide, specifically the B-band, is the major antigen recognized by the immune system. This antigenicity is the basis of the O-serotyping system for classifying Gram-negative bacteria. Currently, 20 O-serotypes in *P. aeruginosa* have been identified⁸². Smooth LPS is also involved in complement activation and inhibition by preventing the membrane attack complex from attaching to the cell. Rough mutants of *P. aeruginosa* are avirulent in animal models of infection indicating that the O-polysaccharide is required for pathogenicity²¹. Interestingly, strains isolated from chronically infected cystic fibrosis patients lack the O-polysaccharide, perhaps in an attempt to avoid detection by the immune system¹⁴.

3. PROTEINS

The outer membrane has a myriad of other functions critical to bacterial physiology. These functions are mediated by different classes of outer

membrane proteins. The lipoproteins OprI, OprL, and the Tol system have structural and membrane stabilization roles, such as the maintenance of cell shape^{63, 91}. Flagella and fimbriae are involved in cellular mobility and adherence. Porins mediate the selective uptake of a number of compounds ranging from small nutrient molecules to larger iron–siderophore complexes. Many antibiotics enter the cell through this route as well. There are also a large number of efflux and secretion systems responsible for exporting toxic compounds, proteins, DNA, virulence factors, and a variety of other substrates from the cell. Because of their location on the cell surface, these proteins, as well as many other outer membrane proteins, can act as adhesins, antigens, and receptors for phages and bacteriocins. We will only discuss the porins, as the other protein classes mentioned above are covered in other chapters. A more detailed discussion of the known outer membrane proteins of *P. aeruginosa* and especially the putative porins is found in Hancock and Brinkman⁴².

3.1. Selective Permeability of the Outer Membrane

The outer membrane of *P. aeruginosa* is a formidable barrier for antibiotics and other large, hydrophobic molecules having a net permeability that is only 1–8% that of *E. coli*³⁸. Only the water-filled channels of porins are available for the passage of small hydrophilic molecules, including substrates and antibiotics. In particular, the porin channels used by *P. aeruginosa* for nutrient uptake are either inefficient or highly specialized, and permit few substrates larger than a monosaccharide (~200 Da) into the cell⁴².

There are four classes of porins. General porins allow the diffusion of a wide range of structurally diverse compounds into the cell. Specific porins facilitate the uptake of unique substrates via stereo-specific binding sites (Figure 2). Gated porins selectively take up large molecules such as iron–siderophore complexes. Efflux porins, or channel tunnels, work in conjunction with inner membrane pump and periplasmic linker proteins to expel toxic molecules from the cell. These latter proteins, forming a family of 18 proteins related to OprM⁴² are covered in more detail in Chapter 21 in this book.

It should be noted that the outer membrane does not completely exclude either small hydrophilic or hydrophobic compounds from the cell. Antibiotics eventually do equilibrate across the *P. aeruginosa* outer membrane, albeit at a much slower rate, than observed with other bacteria. The lower rate of entry ensures that secondary resistance mechanisms such as efflux pump systems (with the major efflux system involved in intrinsic antibiotic resistance being MexAB–OprM for many antibiotics and MexXY–OpmG for aminoglycosides) and β -lactamases work efficiently, and are not overwhelmed by high

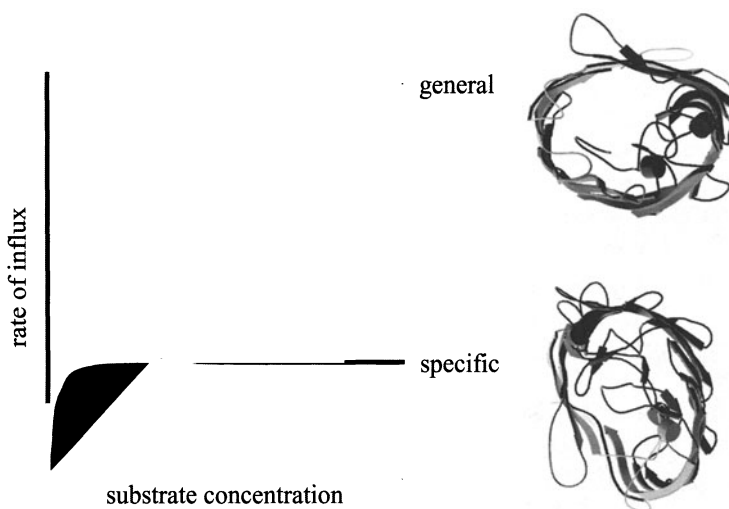


Figure 2. Uptake through general and specific porins. Potential substrates of a given size and charge are able to pass through general porins. The rate of uptake through these porins is proportional to the substrate concentration. Shown here is the top, extracellular view of a monomer of the trimeric PhoE anion selective porin of *E. coli* ref. [19]. Specific porins have substrate-specific binding sites that may be formed in part by extracellular loops that fold into the channel mouth as with the *E. coli* maltodextrins-specific LamB channel ref. [91]. Uptake through specific porins is accelerated at low substrate concentrations and plateaus at substrate concentrations high enough to saturate the binding sites of these channels.

concentrations of antibiotics³⁸. This then provides the major basis for the high intrinsic resistance of *P. aeruginosa* to antibiotics, a major factor in the known clinical resistance to therapy of this organism.

3.2. General Porins

In Gram-negative bacteria, most hydrophilic compounds traverse the outer membrane via non-specific (general) porins. These channels are considered passive since transport is dependent on the solute's physicochemical properties. The diameter of the water-filled space in the most constricted part of the channel determines the size of molecule that can pass through, that is, the exclusion limit. In the prototypic bacterium, *E. coli*, the major porins are OmpF and OmpC and these permit molecules up to 600 Da to diffuse through the outer membrane. The external vestibules of non-specific porin channels are rich in charged amino acids, and there are also charges around the constriction zone⁵⁴, imparting some selectivity based on the charge of the solute. General porins are able to support the rapid influx of solutes in nutrient-rich

conditions. Under these conditions, the incoming molecules can be either rapidly metabolized by enzymes in the periplasm or transported to the cytoplasm via high affinity cytoplasmic membrane transporters, thus maintaining a large concentration differential between the two faces of the outer membrane.

The *Pseudomonads* are different from many Gram-negative organisms because of their lack of general porins with homology to the non-specific porin family. This results in a generally low outer membrane permeability, although in apparent contradiction to the exclusion limit of *P. aeruginosa* which is relatively high (in the order of 3,000 Da)⁹. In *P. aeruginosa*, whose porins have been the most extensively characterized, two porins apparently provide the ability to permit passage of general substrates. OprD, is a specific porin, but appears to be the major conduit for small molecules of less than 200 Da⁴⁸. OprF, a member of the OmpA family of outer membrane proteins has an apparently large exclusion limit, but demonstrates functional heterogeneity with only a small proportion of the OprF molecules forming large channels. It is probably responsible for the permeation of molecules between 200 and 3,000 Da⁹. In addition, OprB appears to be the major conduit for saccharides¹¹⁴. The function of another putative porin, OprG, is not clear. The lack of members of the general porin family in *Pseudomonas* and the relatively limited expression of other porins, also contributes to the high intrinsic resistance these organisms display towards many toxic compounds.

3.2.1. OprF OprF shares C-terminal homology with OmpA and thus is a member of the OmpA superfamily of porins²⁴. It is one of the major *Pseudomonas* outer membrane proteins having a copy number of approximately 200,000 per cell⁸. Because it is so abundant, there has been considerable interest in developing OprF as a vaccine candidate^{20, 86, 107}. In addition, OprF plays a number of roles important for cellular survival.

3.2.1.1. OprF Structure. Cross-linking studies indicate that OprF is an oligomer, possibly a trimer that is associated with both LPS and peptidoglycan⁵. The protein consists of three domains: the N-terminus, a hinge region, and the C-terminus. A three dimensional model of the N-terminus of OprF (the first 160 amino acids) was constructed based on the homologous regions of the N-terminus of OmpA, indicating that this region is an eight stranded β -barrel¹³. This model is in agreement with data obtained by circular dichroism spectroscopy.

A hinge region from amino acid 161 to amino acid 209 joins the C- and N-termini. This hinge and loop region contains multiple proline-alanine repeats and in *P. aeruginosa*, has two disulfide bonds, although this feature is not conserved in other *Pseudomonads*. We have previously suggested that alternative disulphide bonding of these four cysteine residues explains the two alternative forms and channel sizes observed in planar bilayer experiments with OprF⁹.

The C-terminus of OprF shares considerable similarity with that of OmpA (56%). By analogy with certain data for OmpA, it has been proposed that the OprF C-terminus (amino acids 210–326) may be a globular domain that lies in the periplasm since it is involved in peptidoglycan binding. However, there is considerable evidence suggesting that this region, or a portion of it, is surface exposed. First, there is strong bioinformatic evidence indicating that two regions in the C-terminus are β -strands⁴¹. Second, the C-terminus has been used to generate surface reactive monoclonal antibodies⁵⁰ and there have been numerous reports of surface reactive monoclonal antibodies recognizing this region^{20, 108}. Indeed the C-terminal region of OprF is being considered as a component of a vaccine for *P. aeruginosa*. Third, the *P. fluorescens*²³ and *P. aeruginosa* OprF proteins⁷¹ have surface accessible protease cleavage sites. One possibility is that this region can translocate to the surface and that this impacts on channel size. Interestingly OmpA also appears to demonstrate two protein forms with different channel diameters¹⁰⁰.

3.2.1.2. A Structural Role for OprF. *P. aeruginosa* mutants deficient in OprF synthesis have an almost spherical appearance, are shorter than wild-type cells and do not grow in low osmolarity medium¹¹² suggesting that like OmpA, OprF is involved in maintaining cell shape. Analysis of OprF mutants with truncated C-termini showed that it is this region that is involved both in binding to peptidoglycan and the above structural roles⁸⁸.

3.2.1.3. OprF as a General Porin. Planar bilayer methods have shown that *P. aeruginosa* OprF is a non-specific, weakly cation-selective channel with one of two channel sizes. The channels can be either small (0.36 ns) or relatively large (2–5 ns)⁹. This large channel size appeared to some researchers to be in contradiction with the measured low permeability of the *Pseudomonas* outer membrane, and was a major source of controversy. However, intact cell studies involving providing *P. aeruginosa* with a raffinose metabolism system, and measuring the ability to grow on large sugars, as well as plasmolysis experiments, confirmed a large exclusion limit and the role of OprF in determining this property. This dilemma was resolved by the demonstration that only a small proportion of OprF channels (approximately 400 out of 200,000)¹³ form the large size channels and that outer membrane permeability of smaller molecules (the size of most *P. aeruginosa* substrates) is managed by low abundance porins such as OprD and OprB.

Interestingly, the full length OprF protein is required for large pore formation. Mutants with C-terminal truncations in OprF only form the smaller sized pores¹³, suggesting that OprF adopts a different conformation for the larger channel size that probably would involve the participation of the C-terminal half⁸⁸.

3.2.1.4. Role of OprF in Pathogenesis. The OprF proteins from both *P. aeruginosa*⁶ and *P. fluorescens*²³ have both been reported to be involved in adherence to surface receptors in their respective hosts. The homologue in *P. fluorescens* is a fibronectin-binding protein⁸⁹. Recent work has suggested another role for OprF in human infection. Expression of this porin is substantially downregulated in aerobic biofilms compared to anaerobic biofilms, which are presumed to exist in the lungs of infected cystic fibrosis patients. Both OprF and OprF antibodies have been isolated from the mucus of chronically infected patients¹¹⁵. Additionally, OprF expression appears to be under the control of the AlgU regulator that is responsible for alginate production and conversion of *P. aeruginosa* strains to mucoidy³³, as well as the ECF sigma factor SigX¹⁴. OprF mutants form poor anaerobic biofilms. One reason for this defect is that these mutants also do not possess nitrate reductase activity, which is required to form anaerobic biofilms, therefore, it has been proposed that OprF may be required for the uptake of nitrate or nitrite into the cell⁴⁵. Alternatively, the defect may reflect the structural defect in OprF null mutants. These results have reinforced an interest in developing OprF as a vaccine component.

It is not clear whether OprF plays other roles in infection. Clinical isolates that lack OprF and are resistant to multiple antibiotics have been isolated¹⁴. It is possible that OprF is downregulated in response to antibiotic treatment once an infection has been established. This strategy would enable the bacteria to evade the effects of both the immune system and antibiotics.

3.2.2. OprG. OprG was the last major *Pseudomonas* outer membrane protein to be identified. It first drew attention because of its complex regulation. An inverse relationship was noted when *P. aeruginosa* was grown in low iron conditions, therefore it was proposed that this porin functioned in iron uptake. Additionally, OprG expression was affected by entry into stationary phase, higher growth temperatures, low Mg²⁺ concentrations, alterations in LPS structure, and the presence of various carbon sources³⁶. Unpublished results from our laboratory have shown that OprG is regulated by ANR but not DNR suggesting that this porin functions during anaerobic conditions but independent of denitrification (J.B. McPhee and R.E.W. Hancock, unpublished results).

Indeed, attributing a function to this elusive protein has proven difficult. It was proposed that OprG may be involved in fluoroquinolone resistance as increased resistance to norfloxacin can be associated with a loss of OprG. However, these two phenotypes are not obligatorily linked as we have shown that OprG knockout mutants have no antibiotic resistance phenotype. Additionally, despite its similarity to other eight-stranded β -barrels, surface exposed proteins with functions in serum resistance and cell adherence^{16, 109}, no role for OprG in similar activities was detected.

3.3. Specific Porins

In contrast to other Gram-negative organisms that only use specific porins for the uptake of large, bulky substrates such as maltodextrins and nucleotides¹⁰, *Pseudomonas* appear to almost exclusively use specific porins for the uptake of small molecules. There are a large number of specific *Pseudomonas* porins, with many of them having paralogues (between 1 and 18 sequence-related proteins) in the same organism^{73, 99} (www.cmdr.ubc.ca/bobh/omps/). These paralogous families are a unique feature of the *Pseudomonas* and underlie the importance of this class of proteins to these organisms.

Specific porins have saturable stereo-specific binding sites for their substrates and uptake follows Michealis–Menten kinetics. Therefore, uptake is accelerated at low substrate concentrations and plateaus when the sufficient substrate is present to saturate the binding sites. If uptake under nutrient limiting conditions was solely mediated by diffusion, the high affinity active transport systems in the cytoplasmic membrane would be at most 5% saturated, making transport the rate-limiting factor for growth^{47, 74}. Therefore, having specific porins gives organisms a competitive edge in environments deficient in nutrients. In addition, the specific porins of *Pseudomonas* can act as general selectivity filters by taking up low levels of structurally diverse compounds⁴⁸, thus complementing the low uptake activity of the OprF general porin.

3.3.1. Structure of Specific Porins. Despite very little sequence similarity, the primary sequences of porins share several conserved features that result in strikingly similar tertiary structures^{19, 58, 95}. Overall, the sequences are rich in charged amino acids. There are no stretches of hydrophobic residues; instead there are several regions that have 12–25 alternating polar and non-polar amino acids flanked by aromatic residues⁵⁴. In the porins with known structures, these amphipathic regions correspond to the β -strands that make up the walls of the channel, with the aromatic residues anchoring the porin into the membrane. The β -strands are connected at the periplasmic end by short periplasmic turns and at the extracellular surface by longer loops. These loops are the most variable regions among the porin superfamily and they largely determine the properties of the porin. The loops are involved in stabilizing the porin by interacting with LPS and the other porin monomers. As well, they modulate uptake activity by folding into the channel mouth to constrict the opening⁹⁴. The C-terminal β -strand of these porins is the most conserved region among these proteins⁵¹ with the terminal amino acid generally being an aromatic residue. Since these sequence characteristics appear to be shared among the specific porins of *Pseudomonas*, it is assumed that the tertiary structure will be conserved as well.

Cross-linking studies⁵ suggest that *Pseudomonas* porins are composed of three subunits. Topological analysis of these porins, involving insertion and

deletion mutagenesis are consistent with the interpretation that each subunit comprises a β -barrel made up of 16 β -strands^{49, 101}. This is reminiscent of the general porins of other Gram-negative organisms, but in contrast to the structure of the maltodextrins-specific porin LamB, which contains 18 β -strands per monomer.

The substrate-binding site is formed in part by some of the extracellular loops. In several porins, the longest loop, the third one, plays a critical role in substrate binding^{49, 54, 102}. In addition the barrel walls may contain residues that are important for facilitating passage of the substrate, similar to the greasy slide motif of the LamB porin of *E. coli*¹⁰⁶.

3.3.2. *OprB*. In *P. aeruginosa*, glucose and other monosaccharides (xylose, mannitol, fructose, and glycerol) enter the cell via the OprB porin. These porins are also present in *Pseudomonas putida*, *Pseudomonas fluorescens*, *Pseudomonas chloraphis*, and *Pseudomonas syringae* and for the first three organisms have been shown to facilitate the entry of glucose into cell, although their specificity for other carbohydrates differ². OprB is the closest homologue the Pseudomonads have to the maltodextrins-specific LamB porin of *E. coli* and shares some structural features with the latter porin. Modelling of OprB suggests that like LamB, it has 18 β -strands and a cluster of aromatic residues resembling the greasy slide of LamB⁴¹. The aromatic rings in this motif provide Van der Waals contacts for the incoming pyranose rings and guide them through the channel. Circular dichroism spectropolarimetry of the functionally characterized OprB proteins has shown that the β -sheet content in these porins varies from 31% to 50%², suggesting that there may be slight structural differences in these channels that may account for the differences in secondary substrate and selectivity. The difference in ion selectivity among the *P. putida* (cation) and *P. aeruginosa* (anion) OprB channels also suggests that there may be structural variability among these proteins¹¹³.

OprB is positively regulated in response to glucose⁴. In *P. aeruginosa*, the regulation is mediated by a two component regulatory system consisting of the GltR transcriptional activator³ and an as of yet uncharacterized sensor kinase putatively encoded by PA3191. Also, OprB expression is under catabolite repression control and is downregulated in the presence of citric acid cycle intermediates⁴.

Interestingly, both *P. aeruginosa* and *P. putida* have two OprB paralogues. In both organisms, the gene corresponding to the previously studied *oprB* is in an operon with genes encoding homologues of the high affinity glucose uptake system. The newly discovered *oprB* homologues (*opbA* in *P. aeruginosa*⁹⁹ and *oprB2* in *P. putida*⁷³) are downstream of a glucose dehydrogenase gene. Glucose dehydrogenase plays a role in the low affinity uptake pathway for glucose⁶⁷; whether the two OprB homologues are also involved in this pathway, remains to be determined.

3.3.3. *OprP* and *OprO*. *OprP* is the phosphate-specific porin of the Pseudomonads. It is induced under phosphate limiting conditions (i.e., 0.15 mM) by the *PhoB* regulator, which also controls the expression of a periplasmic phosphate binding protein⁴⁴. This co-regulation, together with the fact that an *OprP* knockout mutant is deficient in high affinity phosphate-inducible transport⁴¹ suggests that the transport systems in the outer and cytoplasmic membranes interact to bring phosphate into the cell, although there is no direct evidence supporting this assumption.

The molecular architecture of *OprP* has been probed using a variety of techniques. Predictions based on the *OprP* amino acid sequence as well as insertion mutagenesis suggest that *OprP* has 16 β -strands per monomer¹⁰¹. A lysine residue K₁₂₁ in the proposed loop 3 was shown by site-directed mutagenesis to be involved in phosphate binding and passage through the *OprP* channel. Two other lysine residues, one in loop 3, K₁₂₆, the other in trans-membrane region 4, K₇₄, modulate rather than destroy phosphate binding and thus may represent secondary binding sites¹⁰².

P. aeruginosa has an *OprP* homologue called *OprO* that is 76% similar to *OprP*⁹⁸. The *oprO* gene is immediately upstream of *oprP*. Like *OprP*, *OprO* is induced during periods of phosphate starvation, and presumably regulated by *PhoB*, but it is only produced after growth into stationary phase. Unlike *OprP*, *OprO* preferentially binds pyrophosphate.

In addition to its *OprP* homologue, *P. fluorescens* possesses another phosphate starvation-inducible outer membrane protein called *Ag1*. Based on N-terminal protein sequencing results, this protein is not homologous to any known protein. When resolved on an SDS-PAGE gel, its apparent molecular weight, 55 kDa, is greater than that of *OprP* (48 kDa) and in contrast to *OprP*, its migration is not affected by different solubilization temperatures. Whether this protein is indeed an *OprP* homologue with an unusual N-terminal sequence, or a porin, remains to be determined⁶².

3.3.4. *OprD* Family. The *OprD* porin has been studied quite extensively due to its specific role in antibiotic resistance^{56, 81}. This porin facilitates the passage through the outer membrane of the basic amino acids, lysine and arginine, and small peptides containing these amino acids¹⁰⁵. In addition it is also the major route of entry for the structurally related carbapenem antibiotics imipenem and meropenem. A variety of techniques have shown that these substrates compete for the same binding site in the channel¹⁰⁴. In addition, *OprD* acts as a general porin by permitting the passage of structurally unrelated small molecules such as gluconate into the cell⁴⁸.

OprD shares 15% amino acid sequence similarity with its closest *E. coli* homologue *OmpF*. Alignment of these two proteins suggested that the *OprD* monomer consisted of a 16-stranded β -barrel. This model was tested and refined by constructing *OprD* variants with short 4–8 amino acid deletions in

the putative loop regions^{49, 76}. Further analysis of the deletion mutants has shown that both loops 2 and 3 are involved in substrate binding⁷⁶. Loop 7 may also have a role in substrate binding since the OprD proteins of clinical isolates resistant to meropenem have several amino acid substitutions in this region as well as a two amino acid deletion²⁶. Mutants with deletions in loops 5, 7, and 8 have larger channels and permit the passage of multiple antibiotics⁴⁹. Therefore, these regions are thought to fold into or over the channel and thereby constrict the opening. In contrast to most other porins, the last OprD amino acid residue is a leucine rather than an aromatic residue, but how this contributes to the overall tertiary structure or folding remains to be determined.

The importance of OprD to the cell is highlighted by its complex regulation. It is induced by its substrate arginine via the ArgR regulator. Alanine and glutamate induce *oprD* independently of ArgR through an as of yet uncharacterized regulator⁷⁷. OprD is also under the control of catabolite repression⁷⁸. Additionally, imipenem stimulates the MexT regulator to repress *oprD* levels and induce the *mexEF-oprN* efflux operon, which extrudes carbapenems and quinolones⁵⁵. Salicylate, a compound released by plants upon infection, represses OprD via an unidentified regulator⁷⁸.

The release of relevant genome sequences lead to the finding that OprD is the prototype of a large family of porins. There are 19 OprD homologues in *P. aeruginosa*⁹⁹, 21 in *P. putida*⁷³, 10 in *P. syringae*, and 10 in *P. fluorescens*, plus many more in Pseudomonads whose genomes have yet to be sequenced. The closely related soil bacterium *Azotobacter vinelandii* also boasts a large number of OprD homologues. The genomic context of these homologues indicates their involvement in the uptake of a variety of metabolites. The *P. putida* family has a large number of OprD homologues predicted to take up aromatic compounds. Phylogenetic analysis of the *P. aeruginosa* OprD family shows that members of this family belong to one of two subfamilies⁴². One subfamily contains OprD and the homologues in this group that have been studied are involved in the uptake of amino acids and peptides. The members of other subfamily are most similar to the PhaK, phenyl acetic acid uptake porin of *P. putida*⁸⁰. It was originally predicted that members of this cluster would take up aromatic hydrocarbons. However, subsequent analysis of some of these homologues has shown that they are involved in the uptake of a variety of organic compounds, such as dicarboxylic acids, that better reflect the metabolic capabilities of *P. aeruginosa* (S. Tamber and R.E.W. Hancock, unpublished results).

3.4. Gated Porins: TonB-Dependent Receptors

Iron, an essential cofactor of many proteins involved in aerobic respiration, is an indispensable micronutrient for virtually all life forms. However, this

mineral is often limiting in environmental niches due to the low solubility of the ferric ion, and the high degree of competition from other organisms. The importance of iron to the microbial lifestyle is highlighted by the diversity of iron acquisition strategies they have evolved^{12, 29}. *Pseudomonas* species produce a variety of siderophores, which are large molecules that bind strongly to Fe^{3+} (see refs [18], [72]). These molecules have different affinities for the ferric ion and bind to specific surface receptors/uptake porins, thus allowing organisms to tailor their iron uptake capabilities to their particular niches. In addition, to their own siderophores, *Pseudomonads* can take up heterologous siderophores produced by other bacteria and fungi⁸⁵. For example, *P. aeruginosa* also has the ability to acquire iron directly from haem and haem-containing proteins such as haemoglobin⁷⁹, as well as the *E. coli* siderophore enterobactin.

Siderophore-iron complexes enter the cell through specialized receptor/porins termed gated porins, or IROMPs (iron repressible outer membrane proteins). These gated porins comprise a large family with 35 homologues in *P. aeruginosa*⁹⁹, 29 in *P. putida*⁷³, 23 in *P. syringae*, 26 in *P. fluorescens*, and countless others from *Pseudomonas* species whose genomes have not been sequenced yet. Due to their demonstrated or assumed dependence on a particular energy-transducing system they are called the TonB-dependent family. Generally speaking, it is assumed that each channel is specific for a particular siderophore, explaining the great diversity of gated porins in *Pseudomonas*. This chapter will only cover those TonB-dependent receptors that have been functionally characterized.

3.4.1. Structure of TonB-Dependent Receptors. The crystal structures of two *E. coli* TonB-dependent receptors, FhuA and FecA show that these proteins are monomers consisting of 22-stranded β -barrels^{30, 32}. Near the N-terminus of these channels is a globular domain, termed the plug, which contains a four-stranded β -sheet and four α -helices that form part of the gating mechanism. The plug is held in the opening of the channel by 9 salt bridges and more than 60 hydrogen bonds, and is thought to undergo a conformational change upon substrate and TonB engagement that leads to the opening of the channel³¹.

The crystal structures of the above TonB-dependent receptors both with and without their substrates have been solved and suggest a functional uptake mechanism for this class of proteins. First, the siderophore docks into a binding pocket rich in aromatic residues on top of the plug domain. An arginine residue in the pocket then shifts towards the substrate. This shift results in a greater conformational change throughout the protein culminating in the unwinding of an α -helix at the periplasmic side of the channel and the movement of a peptide segment from the opposite barrel wall. Presumably, these changes serve as a signal to the TonB protein in the periplasm. Once the TonB protein recognizes that the channel is loaded with substrate, it is then proposed to utilize energy to induce

another series of conformational changes that both release the substrate from the binding pocket and produce a translocation pathway^{30, 32, 61}.

3.4.2. Pyoverdinin Receptors. The pyoverdins comprise a large family of siderophores. The basic features of these molecules include a dihydroxy-quinolone moiety linked by either a carboxylic acid or a carboxy-amide to a peptide region that varies among different *Pseudomonas* species. Pyoverdinin has a very high binding affinity for the ferric ion. It is the predominant siderophore of the fluorescent *Pseudomonads* and it is this molecule that gives them their fluorescent green colour^{1, 85}.

P. aeruginosa strains synthesize three different pyoverdinin types, each type having its own receptor²². The type I pyoverdinin receptor, FpvA, has been the most extensively characterized and serves as the prototype for this subfamily. An alignment of the FpvA amino acid sequence against TonB-dependent receptors of other Gram-negative organisms predicts that the FpvA monomer has 26 β -strands³⁵. Two residues in the sixth loop, Y350 and A402, have been implicated in ferric-pyoverdinin binding and uptake⁵³. Also, FpvA possesses a 70 amino acid N-terminal extension that is required for the induction of its own expression as well as that of the pyoverdinin biosynthetic operon⁹⁷.

The uptake mechanism through FpvA differs significantly from the TonB-dependent receptors of other Gram-negative organisms⁹³. In its resting state, FpvA is bound to iron-free pyoverdinin³⁴. The iron-free pyoverdinin is displaced by ferric-pyoverdinin, which then enters the cell. Since FpvA binds both iron-free and iron-loaded pyoverdinin with equivalent affinities, it is believed that TonB mediates the displacement.

The pyoverdinin receptors of *P. putida*, PupA¹¹ and PupB⁵⁷, and of *P. fluorescens*, PbuA⁶⁹ have been cloned and characterized. A pyoverdinin receptor from *P. syringae* has also been described but has not yet been cloned¹⁷.

3.4.3. Other TonB-Dependent Receptors. Generally one TonB-dependent receptor is specific for the uptake of one type of siderophore. Given the large number of siderophores, both endogenous and heterologous, utilized by *Pseudomonas* species, the equivalently large number of TonB-dependent receptors in these organisms comes as no surprise. Some members of the TonB-dependent family are conserved among the *Pseudomonads*, while others are species specific and thus may be involved in the adaptation of these organisms to their particular niches¹⁸.

Many *Pseudomonas* species produce secondary siderophores that have considerably lower affinities for Fe^{3+} than pyoverdinin. Pyochelin, produced by *P. aeruginosa* and *P. fluorescens*¹⁵, binds to iron, cobalt, and molybdenum. This siderophore is taken up by the FptA receptor. In *P. aeruginosa*, pyochelin is involved in the acquisition of iron from transferrin, and FptA has been

implicated in virulence¹¹⁰. Quinolobactin⁷⁰, another siderophore produced by *P. fluorescens* is taken up by a 75-kDa outer membrane protein that is induced by quinolobactin and repressed by pyoverdinin, illustrating the siderophore hierarchy in this organism.

In addition to their own siderophores, Pseudomonads can use siderophores produced by other Pseudomonads, other bacteria, and fungi⁶⁴. Again there is a specific receptor that is induced by and takes up a particular siderophore. Since the energy expenditure required to produce many different siderophores is avoided, the ability to use heterologous siderophores gives bacteria a competitive edge in the environment. However, TonB-dependent receptors can also serve as a point of entry for bacteriocins produced by closely related organisms⁷.

P. aeruginosa can take up enterobactin, the major *E. coli* siderophore, via the PfeA receptor²⁵. PfeA shares 60% sequence identity with the *E. coli* enterobactin receptor, FepA and can complement an *E. coli* FepA mutant. PfeA is unusual among the characterized *Pseudomonas* TonB-dependent receptors because it contains a TonB box. Whether this motif is important for uptake activity has not been determined. *P. aeruginosa* pfeA mutants can still take up reduced levels of enterobactin, indicating that a second enterobactin uptake system exists in this organism⁸⁵. Indeed, there is a candidate protein for this role, PirA (PA0931), which shares 72% sequence similarity with PfeA.

In addition to PfeA and PirA, there are several other paired members of the TonB family in *P. aeruginosa*, *P. putida*, and *P. fluorescens* that exhibit greater than 50% sequence similarity to each other. This redundancy may contribute to the overall flexibility of these organisms, allowing them to optimize uptake in a variety of different niches or take up sequence-related variants of a given siderophore. *P. syringae* also has pairs of redundant TonB-dependent receptors, but to a much lesser degree than the three organisms mentioned above.

Iron can also be acquired directly from haem and haem-containing proteins such as haemoglobin. Two receptors in *P. aeruginosa*, HasR and PhuR, are involved in this process⁷⁹. In addition, the genome encodes two as of yet uncharacterized haem receptors, a HasR homologue, OptI (PA4897, 52% similarity), and PA1302 (57% similar to a haem-utilization protein in *Haemophilus influenzae*). PhuR homologues are found in *P. putida*, *P. fluorescens*, and *P. syringae*.

It is important to note that not all TonB-dependent receptors will be involved in siderophore uptake. For example, the gene for the SftP TonB-dependent receptor of *P. putida* lies in a sulphate ester utilization operon. Therefore this protein is predicted to take up sulphate esters. An sftP knockout mutant is able to grow on sulphate esters however; this is presumably due to the expression of a redundant TonB-dependent receptor⁷⁹. Other unusual TonB-dependent receptor proteins in *P. aeruginosa* and their putative substrates include OprC that is specific for Cu²⁺ (see ref. [42]) and PA1271 that is homologous to the BtuB vitamin B12-uptake porin.

4. CONCLUSION AND FUTURE DIRECTIONS

Several features of the *Pseudomonas* outer membrane have contributed to the success of this genus in inhabiting a wide variety of environmental niches. The negatively charged, gel-like matrix of LPS in the outer leaflet of the membrane presents a considerable barrier for large, hydrophobic compounds entering the cell. However, far from being a static structure, the barrier properties of the outer membrane can be modulated, for example, by altering the structure of LPS in the lungs of cystic fibrosis patients. Lipid modifications are also important for maintaining outer membrane integrity and transport capabilities in various conditions. Characterizing additional LPS and lipid modifications and how these changes contribute to the physiology of *Pseudomonas* will continue to be an important area of future research.

Multiple transport systems highlight the inherent flexibility of Pseudomonads to permit growth on a variety of substrates over a range of concentrations and physiological conditions. The challenge in this area will be to assign functions to the large number of newly discovered *Pseudomonas* transport porins. In addition, elucidating the regulatory networks controlling the expression of these proteins will contribute greatly to understanding how they contribute to the fitness of this diverse group of organisms.

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THE Tol–OprL SYSTEM OF *PSEUDOMONAS*

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1. INTRODUCTION

The cell envelope of gram-negative bacteria constitutes a barrier to the entry of many compounds, including deleterious agents, into the cytoplasm. Our early knowledge about the structure and function of this cell component was based mainly on studies with *Escherichia coli*^{84, 96, 97}. Early attempts to better understand the structure and function of the cell envelope of this microorganism led to the analysis of the genotypic and phenotypic properties of bacterial strains mutated in genes encoding membrane components. An easy way to obtain such mutants was to isolate those *E. coli* strains insensitive to the lethal action of colicins. Colicins are toxins, encoded by plasmids, that are produced by and active against *E. coli* and closely related bacteria^{108, 109}. Independent of their mode of action (nuclease or pore-forming activity), these bacteriocins, which are generally released in large amounts into the extracellular medium, must cross the membrane barrier in order to reach their final targets. The mechanisms by which some of these colicins enter the cells have been extensively studied^{17, 54, 64–67, 118}. First, to bind to the cells, colicins must recognize a specific receptor in the bacterial outer membrane. Bacteria carrying mutations in the genes encoding these receptors do not bind their respective

colicin and are, therefore, insensitive to its lethal action. These mutants are termed *colicin-resistant*. After binding to the surface receptor, colicin is translocated through the cell envelope. Mutants have been isolated, termed *colicin-tolerant*, which allow colicins to adsorb to their outer membrane receptor but are insensitive to their toxic effect. Genetic studies have shown that, apart from specific porins, colicins use two different translocation systems to cross the cell envelope, and based on this they have been classified into two groups^{15, 66, 67, 130}. Group B colicins (B, D, Ia, Ib, M, V, 5, and 10) use the Ton system (composed of the TonB, ExbB and ExbD proteins) for their translocation, and group A colicins (A, E1 to E9, K, L, N, bacteriocin 28b, and cloacin DF13) require the Tol system for their import. These two translocation systems have also been "parasitized" for the entry of single-stranded DNA of some filamentous bacteriophages into the *E. coli* cytoplasm (f1, fd, and M13 utilize the Tol system, and T1 and Φ 80, the Ton system)^{26, 27, 95, 120}. Both systems are widespread among gram-negative bacteria and have been proposed to originate from a common ancestor since some of their components seem to be homologous^{14, 133}. In the bacterial genomes sequenced thus far, the genes encoding the Tol system are clustered and their genetic organization is well conserved, whereas the genes of the Ton system are not always clustered¹¹⁹.

Similarly to *E. coli*, *Pseudomonas aeruginosa* (more than 90% of the strains) also synthesizes various types of chromosomally encoded bacteriocins, namely R, F, and S (for recent reviews, see refs [86], [101], [102]), also called aeruginocins or pyocins (by analogy to colicins). From these, the S-type pyocins are protease-sensitive, colicin-like proteins. In fact, some pyocins belonging to this group (S1, S2, and AP41) show considerable sequence similarity with colicin E2¹¹⁴. There is almost no information on the mechanism of pyocin translocation through the *P. aeruginosa* cell envelope, however, it has been suggested that S1, S2, and S3 pyocins could enter the cells via the Ton system, whereas some experimental evidence indicates that AP41 would use the Tol system³¹. In 1973, Holloway *et al.*⁵² isolated and characterized several spontaneous *P. aeruginosa* mutants tolerant to pyocin AP41, which seemed to be altered in two separated chromosomal regions. Twenty-three years later, Dennis and co-workers³¹ confirmed that one of these strains was indeed mutated in one of the *tol* genes, which made this strain the first reported *Pseudomonas tol* mutant. Since that initial report, investigation on the Tol system of *Pseudomonas* has been rather scarce with most articles appearing after 1996. Nevertheless, they still constitute a major source for comparative analysis with *E. coli*, since there are almost no studies published on the Tol systems in other bacteria^{48, 106, 107, 117}. Tol mutants from different bacteria exhibit a common pleiotropic phenotype, mainly characterized for their various defects at the outer membrane level. The Tol system has been extensively studied in *E. coli*, but apart from its implication in the maintenance of outer membrane integrity, the exact function(s) of this system still remain unknown.

The aim of this chapter is to review the current knowledge of the Tol system in *Pseudomonadaceae*, particularly in *Pseudomonas putida* and *P. aeruginosa*, the only *Pseudomonas* species where this gene cluster has been experimentally examined. Nevertheless, since almost all the currently available data on the membrane organization of the Tol system derive from the extensive studies done with *E. coli* (i.e., cell localization and topology of many of its components, protein interactions within the complex, crystallographic studies, etc.), we will frequently refer to *E. coli* and will apply the relevant data to the *Pseudomonas* system.

2. STRUCTURE OF THE Tol-Pal(OprL) PROTEIN COMPLEX

In *E. coli* and *Pseudomonas*, the Tol system, also named Tol-Pal system (Tol-OprL in *Pseudomonas*), is composed of seven proteins, which are associated with different cellular compartments: three inner membrane proteins (TolQ, TolR, and TolA), two periplasmic proteins (TolB, and Orf2- [YbgF-]), one outer membrane lipoprotein (Pal-OprL in *Pseudomonas*-), and one cytoplasmic protein (Orf1- [YbgC-]). In *E. coli*, numerous experiments such as cross-linking with formaldehyde, coimmunoprecipitations, genetic suppression, and yeast two-hybrid experiments, have shown that this system is organized into two protein complexes (Figure 1): an outer membrane complex made up of TolB and Pal, which also interact with Lpp, OmpA, and the

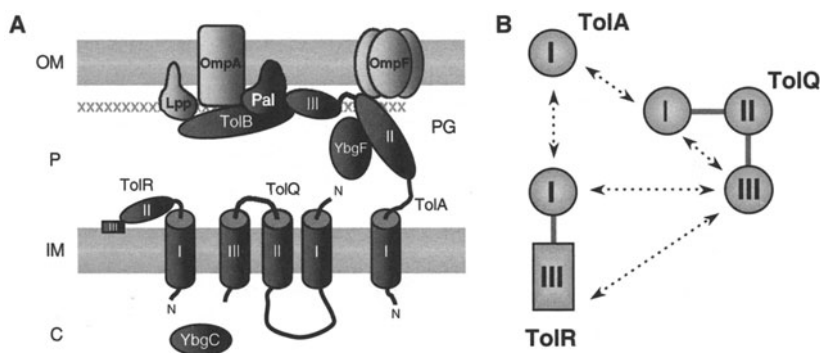


Figure 1. Structure of the *E. coli* Tol-Pal complex. (A) Schematic illustration of the interactions of TolA with Pal, TolB, YbgF (Orf2), and the OmpF trimeric porin, and the interactions of TolB with Pal, Lpp, and OmpA. Components of the Tol-Pal complex are indicated in dark grey. Roman numerals denote different protein domains. C, cytoplasm; IM, inner membrane; N, amino terminus; OM, outer membrane; P, periplasm; PG, peptidoglycan. (B) Diagram of the interactions of the transmembrane segments (circles) of the inner membrane proteins TolQ, TolR, and TolA. The interaction between the TolR C-terminal domain (box), which possesses membrane affinity, and TolQ is also indicated.

peptidoglycan layer^{12, 24, 111}, and an inner membrane complex composed of the TolQ, TolR, and TolA proteins^{21, 32, 41, 73}. YbgF can also be included in this latter complex since its interaction with TolA has recently been demonstrated¹²⁹. These two protein complexes contact each other through the TolA protein in a protonmotive force (pmf)-dependent way^{20, 21, 42}. This confirms the existence of a complex linking the inner and outer membranes, in agreement with previous observations which indicated that the Tol proteins were preferentially localized in the contact regions between membranes⁴⁵.

The cytoplasmic protein YbgC (Orf1) has never been found to interact with any component of the Tol complex, and mutation of *ybgC* in *E. coli* does not confer any obvious distinct phenotype to this bacterium^{23, 121, 129}. The cotranscription of the *ybgC* gene with some of the *tol* genes (see next section) is the only evidence for including YbgC as part of this protein system. Prouty *et al.*¹⁰⁶ claimed that in *Salmonella enterica* serovar Typhimurium, a transposon insertion in this gene conferred a Tol phenotype. However, the analysis of different *tol::luc* reporter fusions, together with the fact that this mutation was 22 bp upstream of the *orf1* ATG start codon, clearly points towards a polar effect of the transposon insertion on *tolR* transcription as the actual cause of this phenotype. In *Pseudomonas*, the *orf1* gene has not been studied, although expression analysis of a His-tagged *P. putida* Orf1 derivative showed that this open reading frame indeed encodes a protein of the expected size (J. J. Rodríguez-Herva, M. Llamas, and J. L. Ramos, unpublished results). YbgC (Orf1) proteins from different bacteria share sequence similarity with the *Pseudomonas* sp. strain CBS-3 4-hydroxybenzoyl-CoA thioesterase⁶, and it has recently been reported that the *Haemophilus influenzae* YbgC protein displays a significant level of thioesterase activity towards short chain acyl-CoA thioesters¹³⁷. However, it is not yet clear how the thioesterase activity of this protein may be related to the function of the Tol–Pal system. The Orf1 proteins are well conserved, albeit less in their C-terminal region, among the four different *Pseudomonas* species analysed in this work (Table 1). The amino acid identity between these and *E. coli* YbgC ranges from 39.2% to 40.7% (Table 1), and the sequence conservation is high within the first third of the protein, particularly in those residues proposed to be part of the active site (including the Asp residue associated with the thioesterase activity)^{6, 137}.

The *E. coli* TolQ, TolR, and TolA proteins are integral inner membrane proteins that interact with each other via their transmembrane domains^{21, 32, 41, 73}. TolQ contains three transmembrane domains and a large cytoplasmic domain between segments I and II¹²⁶ (Figure 1A). TolR and TolA possess a three-domain structure and are anchored to the cytoplasmic membrane by an N-terminal single membrane spanning segment (domain I) that leaves most of the protein exposed to the periplasm^{75, 76, 92} (Figure 1A). The periplasmic part of TolR, that has recently been crystallized,² has two domains: a central domain (domain II)

Table 1. Comparison of the Tol-OprL proteins among the different *Pseudomonas*, and with *E. coli*.

Species compared	Tol proteins						
	Orf1/YbgC	TolQ	TolR	TolA	TolB	OprL	Orf2/YbgF
P-F	76.8	94.4	92.7	77.5	89.1	97.6	80.4
P-S	72.9	93.1	88.3	76.3	87.1	97.6	75.0
P-A	72.7	91.3	82.0	67.4	78.7	91.7	71.4
A-F	74.8	89.6	82.7	63.1	78.1	92.9	69.3
A-S	72.9	90.9	75.3	66.5	77.4	90.5	70.3
F-S	87.1	93.9	85.7	76.5	88.9	97.0	78.2
<i>Ps-E.c.</i>	39.2-40.7	51.5-52.8	30.3-36.5	21.7-25.3	42.2-43.9	39.3-41.6	27.9-30.5

Identity >90%

75%< Identity ≤90%

Identity ≤75%

Values are given as percentage of amino acid identity, and coloured as indicated in the bottom panel. The two values given in the bottom row represent the lower and the higher values of percentage of identity obtained from paired alignments between the *E. coli* Tol proteins and their equivalents in the four *Pseudomonas* species. Alignments were performed with the program CLUSTAL W¹²⁴ using the default settings.

A, *P. aeruginosa*; E.c., *E. coli*; F, *P. fluorescens*; P, *P. putida*; Ps, *Pseudomonas*; S, *P. syringae*.

of about 100 amino acids involved in the dimerization of the protein⁵⁵, and a C-terminal domain (domain III) of about 24 amino acids proposed to form an amphipathic helix that interacts with the cytoplasmic membrane⁷³. The periplasmic region of TolA also contains two domains: a large central domain (domain II) rich in α-helical structures^{34, 75} which interacts with the outer membrane trimeric porins OmpF, OmpC, PhoE, and LamB, but not with the monomeric protein OmpA³³, and a C-terminal domain (domain III). This domain has been crystallized in complex with the g3p protein of the M13 bacteriophage⁸³ and consists of three antiparallel β-strands and four α-helical motifs located on the same side of this β-sheet. Each of the three domains of TolA is separated by a stretch of glycine residues that confers some flexibility to the protein^{34, 75}. A combination of biochemical and genetic studies has shown that the N-terminal transmembrane domain of TolA (I) interacts with the first transmembrane domain of TolQ (I), and with the transmembrane domain of TolR (I)^{32, 41} (Figure 1B). The third transmembrane domain of TolQ (III) interacts with TolR domains I and III, and with its own N-terminal transmembrane domain (I)^{55, 73} (Figure 1B). Although TolQ-TolR dimer interactions have been detected in vivo by chemical cross-linking⁵⁵, there is no experimental evidence demonstrating the formation, even transiently, of a ternary complex involving TolQ, TolR, and TolA.

TolQ is one of the most conserved proteins in *Pseudomonas* and other gram-negative bacteria Tol-OprL systems¹¹⁹ (Table 1). Most likely this is due to its topology and the number of protein interactions in which it participates.

The most conserved regions are those corresponding to the transmembrane segments, especially domains II and III. TolR is less conserved than TolQ (Table 1) and, when compared with *E. coli*, only domains I and III show some significant similarity. TolA shows the least similarity both among the *Pseudomonas* and with the *E. coli* TolA (the similarity to this is basically reduced to the transmembrane helix) (Table 1). In fact, TolA exhibits very poor conservation even between closely related species¹¹⁹. The central α -helical domain (II) of TolA is rich in alanine and charged residues (i.e., in *P. putida* there are up to 10 repetitive elements in this region with the amino acid sequence consensus AKKKAAE(E/D)). The crystal structure of the *P. aeruginosa* TolA C-terminal domain (III) has recently been determined and compared with the structure of the same domain in the corresponding *E. coli* protein¹³³. Although the amino acid sequences of these domains are highly divergent (only 20% identity), their structures were remarkably similar. In addition, data obtained from solution x-ray scattering indicate that the periplasmic domain of *P. aeruginosa* TolA (domains II and III) forms an extended, stalk-like shape. In the crystal structure of the domain III, its N-terminal region forms an elongated α -helix that extends from the compact core of the protein. Witty *et al.*¹³³ have proposed that this helical region forms a helix-helix interaction with domain II to create the elongated stalk, and postulated a fold for the periplasmic domain II + III as an asymmetrical helical bundle with a globular head.

The *E. coli* periplasmic protein TolB⁵³, the three-dimensional structure of which has been determined^{1, 18}, contains an N-terminal α/β domain based on a five-stranded mixed β -sheet that sandwiches two major α -helices, and a C-terminal six-bladed β -propeller domain. It has been shown that TolB dimerizes and that its N-terminal domain promotes this dimerization¹²⁹. Like TolA, TolB interacts with the outer membrane trimeric porins OmpF, OmpC, PhoE, and LamB, and in a Pal-dependent manner with Lpp and OmpA^{19, 24, 112}. The high degree of conservation in TolB among the *Pseudomonas* (except for the N-terminal signal peptide), and with TolB from *E. coli* (Table 1) confirms the importance of its sequence for the preservation of its particular structure.

Pal is an outer membrane peptidoglycan-associated lipoprotein⁷¹. It is anchored to the outer membrane by its N-terminal lipid moiety and strongly interacts with the peptidoglycan layer through its C-terminal region^{11, 60}. The same region of Pal interacts with the β -propeller domain of TolB¹¹¹. The interactions of Pal with TolB and the peptidoglycan appear to be mutually exclusive because the TolB-Pal complex has not been shown to be associated with the peptidoglycan¹¹. It has been demonstrated that Pal also interacts with Lpp and OmpA, and it dimerizes in vivo^{19, 24}. Crystals of a recombinant unacylated Pal protein have been produced and its structure is being determined³. The OprL protein shows the highest degree of similarity (>90% identity) among the *Pseudomonas* Tol proteins. The *P. aeruginosa* OprL protein (formerly

named H or H2) was first characterized by Mizuno and Kageyama^{88, 89} and it constitutes one of the most abundant *Pseudomonas* outer membrane proteins. Interestingly, Hancock *et al.*^{46, 47} have reported the existence of a cross-linked protein complex between the *P. aeruginosa* OprL and OprI lipoproteins (OprI is the equivalent to *E. coli* Lpp, the Braun's lipoprotein), which indicates the level of similarity in the protein interactions that take place in the Tol systems of these two microorganisms. In addition, as in *E. coli*, OprL dimers have been detected *in vivo* in *P. putida* (J. J. Rodríguez-Herva, M. Llamas, and J. L. Ramos, unpublished results). On the other hand, OprL was postulated to be surface-exposed in several gram-negative bacteria, including *P. aeruginosa*, but conclusive proof is currently lacking^{10, 37, 44, 94}. Pal (OprL) proteins are highly immunogenic and are released into serum during gram-negative sepsis contributing to bacterial virulence and inflammation, causing death in mice⁵⁰. In addition, OprL has proven to be a useful target molecule for detection and identification purposes in *P. aeruginosa*^{29, 103}, and for the rapid immunological identification of the pseudomonads belonging to rRNA homology group I¹²³.

E. coli YbgF (Orf2) is a periplasmic protein whose participation in the Tol-Pal system has, until recently, remained a question¹²⁷. However, recent evidence on the interaction between YbgF and domain II of TolA¹²⁹ demonstrates that this protein is indeed an integral part of the Tol-Pal system. In *P. putida* and *P. aeruginosa*, it has been demonstrated that *orf2* is cotranscribed with other genes of the *tol-oprL* cluster^{35, 80}, and its periplasmic localization has been confirmed in *P. putida* (J.J. Rodríguez-Herva, M. Llamas, and J.L. Ramos, unpublished results). Orf2 does not show a high overall similarity among the *Pseudomonas* Tol proteins or when compared with its *E. coli* counterpart (Table 1), and two regions are distinguishable in this protein, with the C-terminal half the most conserved (37.9% average identity with *E. coli*). Orf2 function does not seem to be very important for bacteria, since its gene is absent in some species, like *H. influenzae*^{117, 119}, or interrupted by insertion elements in others (some *P. aeruginosa* strains)⁷⁸.

Finally, it is important to point out that in *E. coli*, the inner membrane TolQRA and the outer membrane TolB-Pal complexes are connected through the interaction of TolA with Pal, and TolB^{20, 21, 42, 129} (Figure 1A). The TolA-Pal interaction requires the pmf, and the TolQ and TolR proteins²⁰. The TolA transmembrane domain, the transmembrane segment of TolR and the third transmembrane domain of TolQ were shown to be involved in the pmf-dependent conformational changes of TolA^{21, 42}. The precise stoichiometry of the *E. coli* Tol-Pal complex is yet to be determined although a model has been proposed to explain the mechanism of the pmf-dependent function of the TolQRA complex²¹. Nevertheless, the stoichiometry appears to be critical for the stability of the system⁸². It is known that Pal is a very abundant protein (30,000–40,000 copies per cell) and that TolA (400–800 copies) and TolR

(2,000–3,000 copies) are minor proteins, while the amount of TolQ is about three times that of TolR^{19, 82}. In fact, the organization of the *tol-oprL(pal)* gene cluster among different bacteria is remarkably conserved, reflecting a functional requirement for the coordinated regulation of expression and stoichiometry of the different components of the protein complex.

3. TRANSCRIPTIONAL ORGANIZATION AND REGULATION OF THE EXPRESSION OF THE *tol-oprL* GENES

The *tol-oprL(pal)* gene cluster consists of seven genes in the order *orf1(ybgC)*, *tolQ*, *tolR*, *tolA*, *tolB*, *oprL(pal)*, *orf2(ybgF)* (Figure 2). In the four *Pseudomonas* species described in this chapter, the genes flanking the *tol-oprL* system are conserved. The gene immediately upstream of *orf1*, *ruvB*, encodes a DNA helicase involved in the processing of recombination intermediates⁵¹, and the open reading frame located just downstream of *orf2* encodes a hypothetical radical activating enzyme (Figure 2). The arrangement of the *tol-oprL(pal)* genes and their intergenic region distances are almost identical within the genus *Pseudomonas*, although their transcriptional organization seems to be different in the two species analysed thus far. While in *P. putida* the *tol-oprL* genes are arranged into two major transcriptional units (*orf1-tolQ-tolR-tolA-tolB*, and *oprL-orf2*)⁸⁰, in *P. aeruginosa* the existence of three transcriptional units (*orf1-tolQ-tolR-tolA*, *tolB*, and *oprL-orf2*) has been proposed³⁵ (Figure 2). On the other hand, in *E. coli*, the *tol-pal* genes are organized into two transcriptional units, *ybgC-tolQ-tolR-tolA* and *tolB-pal-ybgF*^{93, 127} (Figure 2).

In *E. coli* and *P. aeruginosa*, data on the transcriptional organization of the *tol-oprL(pal)* genes are based mainly on analyses of β -galactosidase activity of transcriptional fusions of putative promoter regions to *lacZ*^{35, 127}. Furthermore, most attempts to determine the transcriptional initiation points of the *tol-oprL(pal)* promoters in these microorganisms have failed, possibly due to the low level of transcription and/or the instability and rapid degradation of these mRNA species^{35, 93, 127}. Alternative approaches, such as analysing the mRNA overexpressed from the *tol-oprL* genes cloned into plasmids have usually yielded misleading interpretations^{35, 62}. In *P. putida*, it has been possible to identify the transcriptional start points of the *tol-oprL* gene cluster by primer extension analysis. These were located upstream of the *orf1* and *oprL* genes, respectively⁸⁰ (Figure 2). The *P. putida orf1* promoter is located 91 bases upstream of its translational start codon while the *oprL* promoter is located within the *tolB* gene; 174 bases upstream of the putative *oprL* translational start codon and 120 bases upstream of the *tolB* translational stop codon⁸⁰ (Figure 3). Notably, in

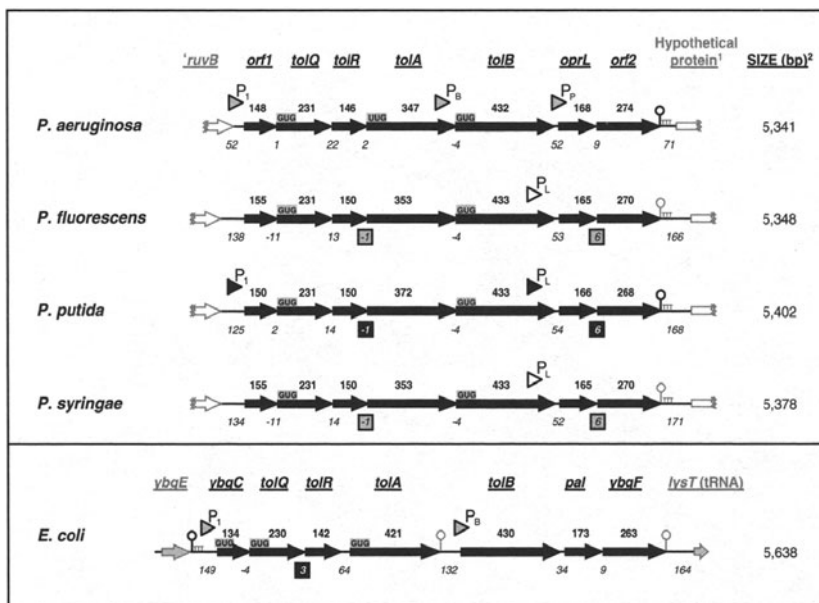


Figure 2. Schematic comparative representation of the DNA regions containing the *tol-oprL(pal)* gene cluster from *P. aeruginosa* PAO1, *P. fluorescens* Pf-5, *P. putida* KT2440, *P. syringae* pv. tomato strain DC3000, and *E. coli* K-12. Black arrows indicate the different *tol* genes, their relative sizes, and the transcriptional direction. The flanking genes (complete or truncated) are indicated in grey (filled or empty arrows, respectively). Numbers in bold type above the genes represent the predicted length (in amino acids) of their corresponding products. Alternative start codons for those genes that do not start with AUG, are indicated in grey boxes above the genes. Numbers in italic type below the map indicate the distance (in base pairs) between adjacent genes. Negative numbers denote that the genes overlap by the indicated number of base pairs. When these numbers are boxed it indicates a theoretically predicted (grey boxes; see Figure 4) or experimentally determined (black boxes) translational coupling phenomenon. Putative transcriptional terminators are indicated by circles above the sequences. Those proposed to be Rho-independent are labelled with an adjacent stretch of Ts. Terminators labelled in black indicate that experimental evidence supports their existence, otherwise (grey ones) they are only predictions based on the nucleotide sequence. Arrow heads above the gene clusters denote the presence of a promoter region as determined by primer extension analysis (black arrows), β -galactosidase gene fusion analysis (grey arrows), or as predicted by sequence similarity (empty arrow.). (1) This hypothetical protein, a probable radical activating enzyme, is well conserved (>71% global similarity) among the four *Pseudomonas* strains analysed. In the *P. aeruginosa* genome, this protein (annotated as PA0975) carries a putative 49 N-terminal amino acid extension absent in the other three strains. Assuming that the gene encoding PA0975 starts in the same AUG position in the four species (which would result in >87% global similarity among them), the intergenic distance between this gene and *orf2* would be 218 bp. (2) The sizes are calculated from the *orf1/ybgC* start codon to the *orf2/ybgF* stop codon. The *tol-oprL(pal)* DNA sequences have been obtained from their respective sequenced genomes (in the case of *P. fluorescens* Pf-5, it has been assembled from different DNA contigs deposited in the TIGR database in April 2003).

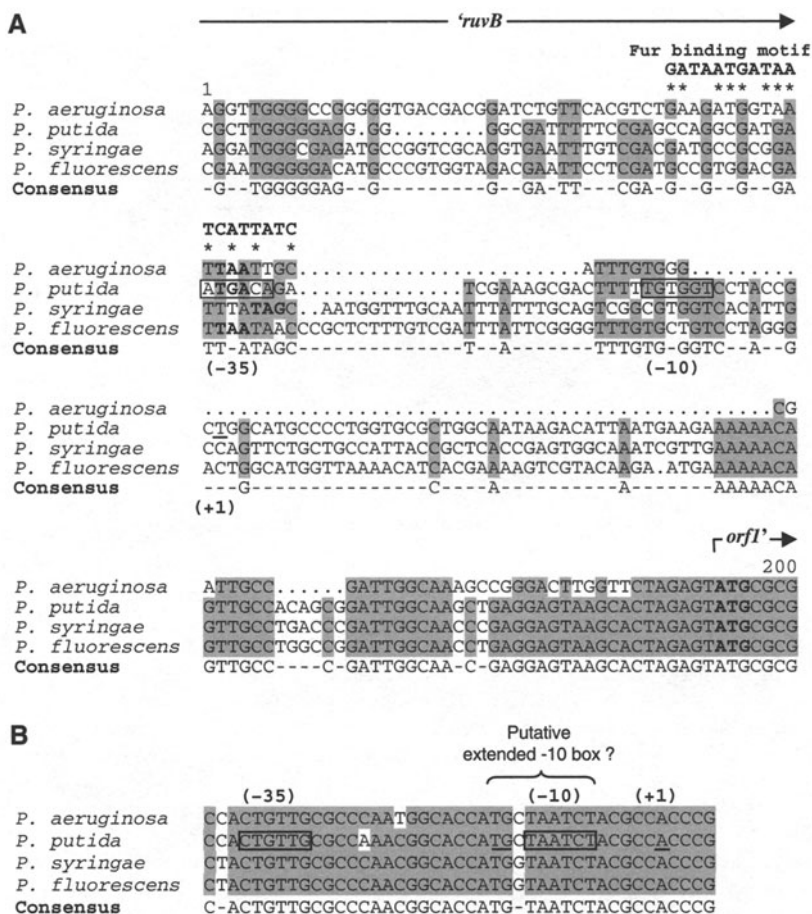


Figure 3. Alignment of the regions containing the *P. putida* KT2440 *tol-oprL* promoters with the corresponding regions from *P. fluorescens* Pf-5, *P. aeruginosa* PAO1, and *P. syringae* pv. tomato strain DC3000. Nucleotides that are conserved at least in three of the four positions are included in the consensus sequence. The predicted *ruvB* stop codons are indicated in bold type. The start positions of the *P. putida* mRNA transcripts (indicated by +1) are underlined. The proposed -10 and -35 promoter regions are boxed. (A) Alignment of the *orf1* promoter region. The consensus motif for ferric uptake regulator (Fur) binding is shown above the empirically determined Fur-box for *P. aeruginosa*⁹⁹. Identical nucleotides between both sequences are indicated by asterisks. (B) Alignment of the *oprL* promoter region. The presence of a 5'-TG-3' sequence (underlined) 1 bp upstream of the proposed -10 hexamer (boxed) would result in a putative extended -10 box. (Modified from an earlier version⁸⁰, with permission.)

H. influenzae, transcriptional analysis of the *P6 (pal)* gene revealed that its transcription initiation point gene was also located within the region immediately upstream from the *tolB* gene (83 bases upstream of the *tolB* translational stop codon)¹¹⁷. Both *P. putida* promoters show features typical of promoters recognized by sigma-70 (Figure 3). However, the -35 box of the *P. putida oprL* promoter exhibits low similarity to the -35 consensus box of promoters recognized by sigma-70, but its -10 box, possibly an extended -10 box (Figure 3B), shows a better match with the *E. coli* consensus than the -10 box of the *P. putida orfI* promoter⁸⁰ (Figure 3A). This could also explain the lower activity of the *P. putida orfI* promoter in comparison to the *oprL* promoter⁸⁰. The different expression levels from these two promoters are in agreement with the situation found in *P. aeruginosa* and *E. coli*. In *P. aeruginosa*, the level of expression of an *oprL::lacZ* transcriptional fusion was higher than that observed for an *orfI::lacZ* fusion³⁵. This was also the case in *E. coli*, where the amount of *orfI* mRNA detected by northern blot analysis was lower than that of the *tolB* mRNA⁹³. Interestingly, Cascales *et al.*¹⁹ recently estimated that in *E. coli*, the Pal lipoprotein is present in larger amounts than the TolA and TolR proteins.

Comparison of the *ruvB-orfI* intergenic regions of the different *Pseudomonas* species analysed (Figure 3A) showed a low level of sequence conservation in the positions where the *P. putida orfI* promoter was found. In addition, in *P. aeruginosa*, this region (52 bp in length) is considerably shorter than in the other three species (*P. putida*, 125 bp; *P. syringae*, 134 bp; and *P. fluorescens*, 138 bp) (Figure 2). Comparison of the *oprL* promoter region revealed, however, a complete conservation of the bases located in the proposed -35 and -10 boxes (Figure 3B). Nevertheless, it is also important to indicate that, since this region is located within the coding sequence of the *tolB* gene, a stronger selective pressure will operate in maintaining the sequence conservation at these positions (although the conservation of the TolB proteins at the amino acid level in this region is not particularly high among the different gram-negative bacteria). Interestingly, the proposed -10 (5'-TAATCT-3') and -35 (5'-CTGTTG-3') regions of the *oprL* promoter, share a great similarity with those proposed for the constitutive sigma-70 promoters from the *P. putida* WCS358 *pfrA*¹²⁵ and *P. aeruginosa algQ* genes⁶¹ (which possess identical -10 [5'-TAATCT-3'] and -35 regions [5'-TTGATG-3']).

In all *Pseudomonas* species analysed, a well-conserved putative Rho-independent transcriptional terminator (ΔG_0 ranging from -18.0 to -19.5 kcal/mol depending on the species) is found downstream of *orf2*, the last gene of the cluster (Figure 2). This terminator appears to be functional, based on RT-PCR analysis, at least in *P. putida* and *P. aeruginosa*^{35, 80}. No additional transcriptional terminators are predicted within these gene clusters, making it possible that transcription from the *orfI* promoter can be read through the *tolB* (in the case of *P. aeruginosa*) and *oprL* genes. In fact, in both bacteria, RT-PCR

and β -galactosidase assays strongly support this hypothesis, suggesting the existence of a transcript that is initiated at the *orf1* promoter and would cover the entire *tol-oprL* region^{35, 80}. A similar phenomenon was observed in *E. coli*, where the *ybgC(orf1)* promoter is able to direct the transcription of the whole *tol-pal* cluster, despite the possible presence of a Rho-dependent terminator ($\Delta G_0 = -16.8$ kcal/mol) between *tolA* and *tolB* (Figure 2)^{93, 127}. As Vianney *et al.*¹²⁷ pointed out for *E. coli*, the ability to express *tolB* (in the case of *P. aeruginosa*) and *oprL-orf2* from their own promoters would allow the remaining *tol* genes to be separately modulated. In any case, the putative long *orf1(ybgC)-orf2(ybgF)* mRNA transcript in *E. coli* and *P. aeruginosa* must be highly unstable, as suggested by the fact that only mRNA species smaller than those predicted were detected by northern expression analysis^{62, 93}.

On the other hand, the existence of translational coupling between the *tolR* and *tolA* genes, and the *oprL* and *orf2* genes, has been demonstrated in *P. putida*⁸⁰. A similar situation was also reported for the *E. coli tolQ* and *tolR* genes¹²⁷. Translational regulation is potentially important for the balanced synthesis of these proteins, which is probably required to maintain the stoichiometry of this protein complex. In the case of *P. putida*, analysis of the DNA sequence upstream of *tolA* revealed a potential loop structure in the mRNA region, containing the stop codon of *tolR*, as well as the potential ribosome-binding site and the start codon of *tolA* (Figure 4A). Furthermore, another potential mRNA loop structure was predicted between *oprL* and *orf2* (Figure 4A). Nucleotide sequence comparisons (Figures 4B and C) and mRNA folding analysis (not shown) of the aforementioned intergenic regions with their equivalents in other *Pseudomonas* species revealed that, with high probability, similar mRNA loops would form in the case of *P. fluorescens* and *P. syringae* (with comparable predicted ΔG_0) but not in *P. aeruginosa*. Interestingly, the *P. aeruginosa tol-oprL* system seems to diverge from the other three in certain characteristics (see Figure 2 and below). Although the molecular mechanism of these translational couplings in *P. putida* has not been analysed in detail, the presence of recognizable ribosome binding sites upstream of both the *P. putida tolA* and *orf2* genes⁸⁰ suggests that stable mRNA secondary structures (Figure 4A) would mask the ribosome binding sites of these genes making their translation dependent on the prior translation of the gene located immediately upstream.

Regarding the expression of the *tol-oprL* genes, in *P. aeruginosa* it has been shown that the expression of the *orf1-tolQ-tolR-tolA* gene cluster is negatively regulated throughout growth, by the presence of iron in the culture medium³⁵. In this process the ferric uptake regulator (Fur) was shown to be involved by directly binding to the *orf1* promoter region, where a *fur* box motif is present⁹⁹ (Figure 3A). Iron regulation of the *P. aeruginosa tolB* and

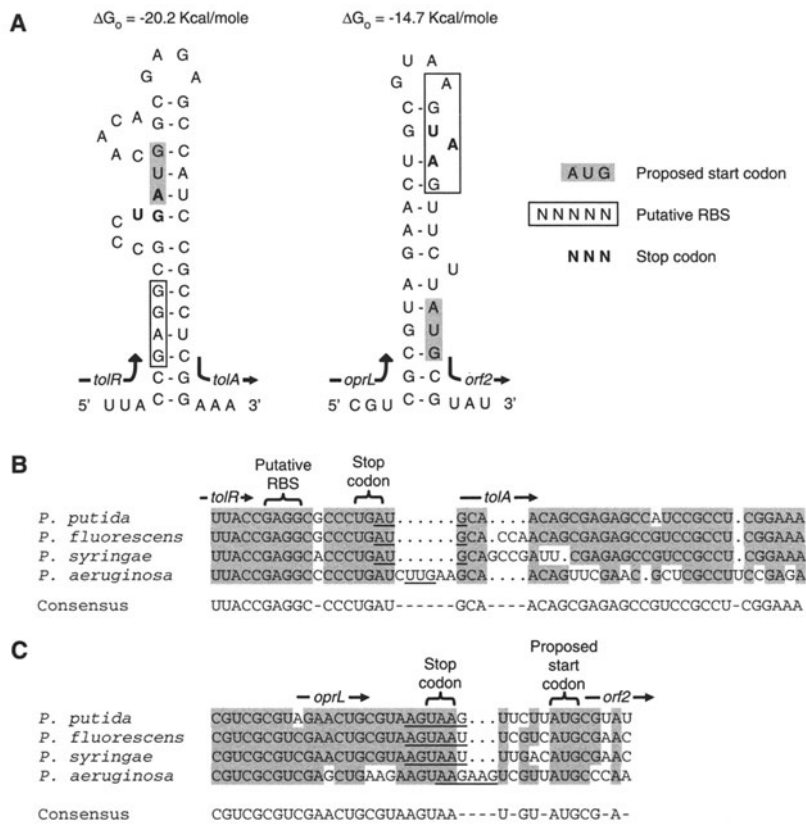


Figure 4. (A) Predicted secondary structure of the *P. putida* KT2440 *tolR*–*tolA* mRNA (left) and *oprL*–*orf2* mRNA (right). The translational stop codons, predicted ribosome binding sites and start codons, are labelled as indicated. The most stable secondary structures and their ΔG_0 were predicted using the *mfold* v2.3 program¹³⁸, with the default settings, and at 30°C. (Reproduced from ref. [80], with permission.) (B) Alignment of the *P. putida* KT2440 mRNA region containing the *tolR*–*tolA* genes with the corresponding regions from *P. fluorescens* Pf-5, *P. aeruginosa* PAO1, and *P. syringae* pv. tomato strain DC3000. Nucleotides that are conserved in at least three of the four positions are included in the consensus sequence. The *tolR* stop codons and the predicted ribosome binding sites for the *tolA* genes are indicated. The proposed *tolA* start codons are underlined. Note that the *P. aeruginosa* *tolA* gene would presumably start at the underlined UUG codon. (C) Alignment of the *P. putida* KT2440 mRNA region containing the *oprL*–*orf2* genes with the corresponding regions from *P. fluorescens* Pf-5, *P. aeruginosa* PAO1, and *P. syringae* pv. tomato strain DC3000. Nucleotides that are conserved in at least three of the four positions are included in the consensus sequence. The *oprL* stop codons and the proposed *orf2* start codons are indicated. The predicted ribosome binding sites for the *orf2* genes are underlined.

oprL-orf2 expression has also been observed, but only in the late logarithmic phase of growth³⁵. While the Fur protein also seems to be involved in this regulation, its participation appears to be indirect, as there are no *fur* boxes in the *tolB* or *oprL* promoter regions³⁵. RegA, an iron-regulated transcriptional activator involved in the expression of the *P. aeruginosa* exotoxin A gene, was also found to participate in the regulation of *tol-oprL* expression. Although this regulator is not essential for the expression of the *P. aeruginosa tol-oprL* genes, it increases their expression in the stationary phase of growth under iron-restricted conditions³⁵. However, there is still a level of iron-regulated expression of the *P. aeruginosa tol-oprL* genes that is not due to either Fur or RegA, which suggests the involvement of other regulatory factors. In *P. putida* no influence of iron concentration on the expression of the *tol-oprL* genes has been observed⁸⁰, in agreement with the fact that no recognizable *fur* box motifs were found in the corresponding promoter regions (Figure 3A). In the case of *P. syringae* and *P. fluorescens*, DNA sequences (matching 9 out of 19 bp in the Fur-binding consensus sequence) have been found in exactly the same location as in *P. aeruginosa* (Figure 3A). Although these are only putative *fur* boxes and require experimental confirmation, it is noteworthy that DNA sequences exhibiting homology of only 10 bp to the consensus have been identified as being directly bound by *P. aeruginosa* Fur⁹⁹.

Another factor that influences *tol* expression in *P. aeruginosa* is temperature. Expression of *tolQ*, *tolR*, and *tolA* is optimal at 37°C and there is a 40–50% reduction at either 25°C or 42°C⁶². A similar situation has been described in *E. coli*, where the expression of *tolR*, *tolA*, and *tolB* genes is between two- and threefold higher when cells are grown at temperatures of 20°C or 25°C²⁵. In *E. coli*, moreover, the expression of the *ybgC-tolQ-tolR-tolA* operon, but not that of the *tolB-pal-ybgF* genes, seems to be regulated by the protein RscC. RcsC is the inner membrane sensor element of the two-component RcsC/RcsB regulatory system controlling the synthesis of the colanic acid capsular polysaccharide¹³². Clavel *et al.*²⁵ described a mutant RcsC protein that led to a high level of capsule expression and decreased *ybgC-tolQ-tolR-tolA* expression (~50%). Based on this evidence, it has been proposed that RcsC modulates, in an opposing manner, the phosphorylated forms of RcsB and an uncharacterized regulatory protein involved in the control of the *ybgC-tolQ-tolR-tolA* genes. In *S. enterica* sv. Typhimurium, however, Prouty *et al.*¹⁰⁶ reported that the RcsCB system does not appear to regulate *orf1* or *tolQ* expression.

Finally, a surprising regulatory mechanism has also been found to affect the functioning of the *S. enterica* sv. Typhimurium Tol–Pal system. Dam methylation in this microorganism apparently plays an important role in controlling the association of Pal, TolB, OmpA, and Lpp with the peptidoglycan in actively growing bacteria, since a substantial decrease in the proportion of these proteins that associate with peptidoglycan was observed in a *dam* mutant in the exponential

phase¹⁰⁷. However, the levels of *tol-pal* mRNA transcripts detected in the *dam* mutant were similar to those found in wild type¹⁰⁷, suggesting the existence of post-transcriptional regulatory mechanisms acting on TolB and Pal, controlled by methylation. Alternatively, these authors suggest that Dam methylation could control the synthesis of other unknown proteins required for the proper association of TolB and Pal with the peptidoglycan. This kind of regulation was not found, however, in *E. coli*¹⁰⁷. These authors also reported for the first time the firm association between TolB and the peptidoglycan. In *E. coli*, it is known that TolB interacts with the main peptidoglycan-associated outer membrane proteins and part of it is recovered with the membrane fraction^{24, 53}. However TolB was never found to strongly interact with the peptidoglycan in this bacteria. Surprisingly in *Salmonella* the situation appears to be different.

4. COMPARISON OF THE Tol-OprL(Pal) AND Ton SYSTEMS

The TolQ-TolR-TolA protein complex of the Tol-Pal(OprL) system has several similarities with the ExbB-ExbD-TonB inner membrane complex from the Ton system, which is involved in the uptake of iron-siderophore complexes and vitamin B₁₂^{13, 56, 90}. The ExbB and ExbD proteins are structurally and functionally homologous to TolQ and TolR, respectively, and in both cases they seem to adopt similar membrane topologies³⁶. In fact, their similarities are high enough to allow some interchange of components and cross-talk between the two systems as was reported for *E. coli*^{14, 59} and even between *E. coli* and *P. aeruginosa*³¹. Due to this cross-complementation phenomenon, the effects of *exbB* or *exbD* mutations are more pronounced in a TolQ or TolR deficient strain, respectively. However, this is not a general fact, as while *E. coli exbB* mutants only exhibit a partial Ton phenotype (inability to grow under iron-limiting conditions), *P. putida exbB* mutants show no growth on those conditions, suggesting that TolQ is unable to functionally replace ExbB in *P. putida*⁹. The *tolQ* and *tolR* genes are often annotated in the genome databases as *exbB* or *exbD* homologues, respectively, and indeed, in certain cases, appear more similar to these genes than to the other *tolQ* or *tolR* genes, despite being located within a *tol-pal(oprL)* gene cluster¹¹⁹. In all genomes analysed, the genes were present as a pair, in the order *exbB-exbD*, and in many cases (~50%), they were found associated with putative *tonB* genes¹¹⁹. Multiple copies of these genes are found in many bacterial genomes. In the *P. aeruginosa* genome, two different sets of *exbB-exbD* and *tonB* genes are annotated, although they are adjacent in only one case^{104, 135}. In *P. putida*, only one *exbB-exbD-tonB* copy is present, arranged into an operon^{9, 43}, while in *P. syringae*, three copies of each gene are annotated.

TolA and TonB proteins are proposed to have similar elongated conformations, spanning the periplasm, but with different structures: TolA exhibits an α -helical structured central domain (domain II) while TonB has a stretch of X-Pro repeats^{75, 105}. The TolA and TonB proteins share a significant sequence similarity in their N-terminal transmembrane anchor domain⁵⁹. In fact, in *E. coli*, replacement of the TonB transmembrane segment by the corresponding TolA domain produces a TolQ–TolR-dependent chimera with TonB function⁵⁸. TolA and TonB proteins from different microorganisms also show some degree of sequence similarity in their C-terminal domains, that is, between the *E. coli* TolA and the *Haemophilus ducreyi* TonB protein⁸³, and between the *H. influenzae* TonB and the *P. aeruginosa* and *P. putida* TolA proteins⁸². Chang *et al.*²² demonstrated, however, that the structures of the C-terminal domains of the *E. coli* TonB and TolA proteins are completely different. Interestingly, the recent crystallization of the *P. aeruginosa* TolA C-terminal domain and its comparison with the C-terminal domain of the *E. coli* TonB protein has revealed an unexpected structural relationship. The structure-based alignment between these two proteins has revealed a match of the highly conserved loop elements, in particular a Pro–Asp–Gly motif at the first tight β -turn, and a correspondence of the secondary structural elements¹³³. Based on this structural similarity, the authors propose that the TolA and TonB C-terminal domains share a common ancestral fold and could be related by means of domain swapping.

The Ton complex functions as an energy transduction system that couples the energy from the cytoplasmic membrane to the outer membrane for the active transport of iron–siderophore complexes and vitamin B₁₂ through specific outer membrane receptors^{13, 56, 57, 74, 90}. The gated receptors are opened upon ligand binding in a process that requires TonB conformational changes which, in turn, are dependent on the pmf, ExbB, and the ligand binding to the receptor⁶³. However, it is currently not clear how the pmf drives the protein–protein interactions and how the gradient triggers the transport process. As mentioned above, energy-dependent conformational changes in TolA, which require the pmf, and the TolQ and TolR proteins, have recently been characterized in *E. coli*^{21, 41}. Thus, TolA also appears to be analogous to the TonB protein, mediating pmf-dependent interactions between cytoplasmic membrane and outer membrane components. In this system, TolA would initiate a signal to Pal, via a conformational change, generating a transient interaction between the inner and outer membrane Tol complexes. However, the purpose of these interactions is still unknown. Finally, TolQ, and TolR also show structural and functional homologies with MotA and MotB, components of the flagellar motor, raising the attractive hypothesis that the transmembrane domains of TolQ, TolR, and TolA constitute an ion potential-driven molecular motor²¹. In addition, MotB also contains the conserved peptidoglycan-associating motif characteristic of Pal and OmpA⁶⁰.

5. FUNCTION OF THE Tol–OprL(Pal) SYSTEM

As mentioned in Section 1, some colicins and pyocins “parasitize” the Tol–OprL(Pal) system to enter the bacterial cells^{31, 66}. The involvement of different proteins from the Tol system in the translocation of group A colicins has been extensively studied and has recently been reviewed⁶⁷. The single-stranded DNA of some filamentous bacteriophages also use the Tol pathway for entry into the bacteria^{49, 120, 121}. In fact, the Tol system has been proposed as a model to understand the mechanisms used by large molecules to cross the bacterial cell envelope⁶⁵. Apart from its involvement in the translocation of these macromolecules, other roles have been proposed for the Tol–OprL(Pal) system based on the phenotypes exhibited by the *tol* mutants. Mutants in the *tol-oprL(pal)* genes from different bacteria exhibit a similar pleiotropic phenotype: release of periplasmic and outer membrane proteins into the extracellular medium, bleb formation at the cell surface, increased sensitivity to a variety of deleterious compounds, impaired utilization of a number of carbon sources, and in some cases, filament formation and a mucoid phenotype. The wide distribution of the *tol-oprL(pal)* gene cluster in gram-negative bacteria and the conservation of its gene organization, together with the inability to construct *tol* mutants in some species, suggest an important role for the products of these genes¹¹⁹. Nevertheless, aside from its implications in the maintenance of outer membrane integrity, the precise physiological function(s) of these proteins is currently unclear, although, a number of different roles have been proposed.

5.1. Outer Membrane Stability

Increasing evidence indicates that the Tol–OprL(Pal) system is involved in the maintenance of outer membrane stability. It has been demonstrated that mutations in the *tol-oprL(pal)* genes of *E. coli*, *P. putida*, *P. aeruginosa*, *Vibrio cholerae*, or *S. enterica* sv. Typhimurium cause hypersensitivity to different harmful compounds such as detergents, solvents, and various drugs (i.e., EDTA, SDS, deoxycholate, and some antibiotics)^{48, 70, 79, 87, 106, 110, 130}. These mutants also release periplasmic proteins into the growth medium^{7, 48, 79}. In fact, a number of *tol* mutations were originally isolated as periplasmic-leaky (*lky* or *exc*) mutations^{38, 68, 72}. They also show extensive blebbing on their cell surface, especially mutants in the *tolQ*, *tolR*, or *tolA* genes^{7, 79} (compare Figures 5A and B), although it is still unclear whether the periplasmic leakage results from the outer membrane vesicle formation. The *tol-oprL(pal)* mutations also cause cell filamentation in some bacteria, like *P. aeruginosa*⁵², *P. putida*⁷⁹ (Figures 5B and C), and *V. cholerae*⁴⁸. In *E. coli*, a *tolA* mutation

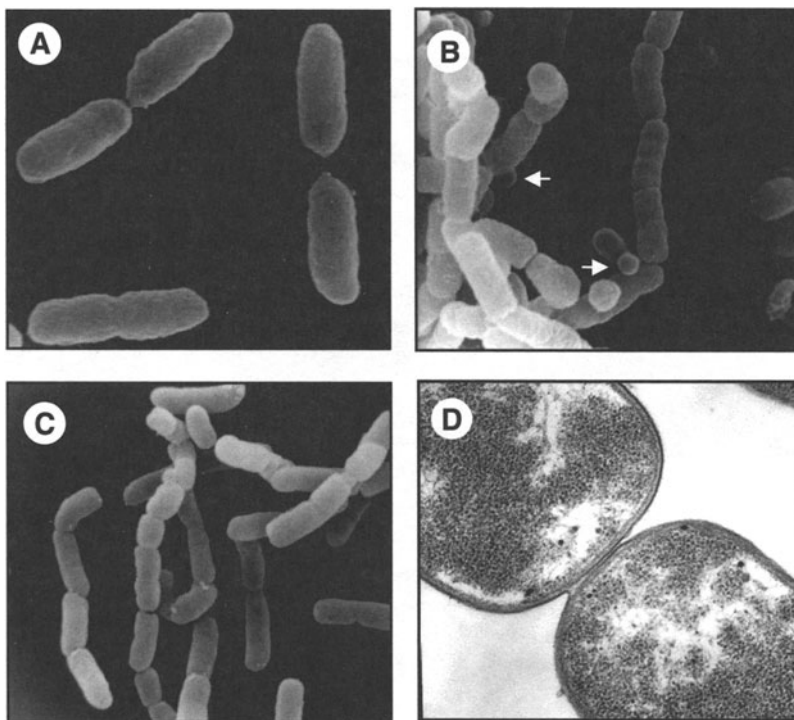


Figure 5. Electron micrographs of *P. putida* KT2440 and some of the *P. putida* *tol* mutants. Scanning electron micrograph of (A) *P. putida* KT2440. Magnification, $\times 15,000$; (B) *P. putida* *tolR::ΩKm*. Magnification, $\times 10,000$ (arrows indicate outer membrane blebs); and (C) *P. putida* *oprL*. Magnification, $\times 7,000$. (D) Transmission electron micrograph of *P. putida* *oprL* showing in detail the division septum between cells which belong to a longer filament. Magnification, $\times 50,000$. In all cases, cells were grown on LB medium and examined when they reached the exponential phase of growth. (Reproduced from ref. [79], with permission.)

impairs septation and cell division^{40, 85}. In *P. putida*, division septa are usually easily distinguishable within cell chains, and in many cases the cell division process appears to be affected at a very advanced stage⁷⁹ (Figure 5D). Furthermore, these filamented cells were non-motile^{40, 52, 79}. Membrane perturbations similar to these have only been observed in *E. coli* mutants altered in the structural protein Lpp^{39, 122}.

In addition, some *tol-pal* mutations lead to a mucoid phenotype. Bernstein *et al.*⁸ first reported that colonies of some *E. coli* *tolB* and *tolA* mutants were mucoid when grown at low temperature on minimal medium. The mucoid phenotype differs depending on the specific type of *tol* mutation since some *E. coli* *tol-pal* mutants are mucoid independently of the growth

temperature and culture medium²⁵. This phenotype is due to the RcsCB-dependent activation of the *cps* genes involved in capsule synthesis²⁵. Although the exact external signal recognized by the RcsC protein remains unknown, it has been proposed that it could sense perturbations on the cell surface (such as those produced by the *tol-pal* mutations) and, as a consequence, would activate capsule synthesis to protect the cell. In *S. enterica* sv. Typhimurium, it has been reported that mutations in the *orf1(ybgC)*, *tolQ*, or *tolR* genes do not provoke a mucoid phenotype¹⁰⁶. However, it has recently been demonstrated that a *tolB* mutation in *S. enterica* sv. Typhimurium promotes *cps* transcription in an *rcsA*- and *rcsB*-dependent manner⁹¹.

Whiteley *et al.*¹³¹ have recently reported that the expression of the *P. aeruginosa* *tolA* gene increases 3.9-fold when planktonic bacteria adopt a sessile biofilm lifestyle, and they suggest a possible contribution of the TolA protein to the increased antibiotic resistance observed in bacterial biofilms.

All these data, together with the fact that TolB and Pal interact with two of the main peptidoglycan-associated outer membrane proteins (Lpp and OmpA), argue in favour of a structural role for the Tol–Pal(OprL) system in the scaffolding of interactions between the outer membrane and the peptidoglycan. Cascales *et al.*²⁰ have proposed that the phenotype of *tol-pal(oprL)* mutants could be a consequence of the desynchronization of outer membrane and peptidoglycan synthesis due to the absence of a correct structural link between the two components.

Does the Tol–OprL system play a role in outer membrane vesicle formation? Many gram-negative bacteria produce outer membrane vesicles during normal growth which have the potential to kill other bacteria, giving them a survival advantage in certain environments^{77, 136}. Bacterial species known to possess an outer membrane instability leading to the production of large number of outer membrane vesicles have been postulated to lack a functional Tol–OprL(Pal) system¹¹⁹. This, together with the fact that *tol* mutants show significant outer membrane blebbing (Figure 5B), has led to the suggestion that this system could also be involved in controlling the formation of naturally produced membrane vesicles⁶⁷. However, this putative function is probably just an indirect effect of the role of these proteins in the maintenance of outer membrane integrity.

5.2. Uptake of Solutes Across the Cytoplasmic Membrane

Aside from the implication of the Tol–Pal(OprL) system in the maintenance of outer membrane integrity and cell morphology in gram-negative bacteria, an additional and unexpected role for this system in the transport of various carbon sources across the cytoplasmic membrane has recently been described⁸¹. Mutations in the *tol-oprL(pal)* genes of *P. putida*, *P. aeruginosa*,

and *E. coli* lead to the impaired utilization of a number of carbon sources by these bacteria⁸¹. Uptake experiments carried out with spheroplasts, which lack the outer membrane, using radiolabeled compounds, strongly suggest that the *P. putida* Tol system influences the transport of certain carbon sources across the cytoplasmic membrane⁸¹. The *P. putida* and *P. aeruginosa tol-oprL* mutants are not able to grow in carbon sources such as arginine, fructose, glucose, glycerol, proline, succinate, or sucrose, which are transported by transport systems of very different nature⁸¹. While glycerol uptake involves a facilitated diffusion process down a concentration gradient that does not require energy^{115, 116}, the uptake of proline is mediated by the PutP protein, which is an energy-dependent Na⁺-symporter^{16, 128}, and the transport of arginine and glucose are mediated by a periplasmic-binding protein-dependent ABC transport system that requires ATP as an energy source^{4, 98}.

A reduced utilization of some carbon sources by certain *tol-pal* and *lky* (putative *tol*) mutants had previously been observed in *E. coli*, but this phenotype was ascribed to the loss of the periplasmic binding proteins involved in the transport of some carbon sources^{5, 69, 72}. The *P. putida tol-oprL* mutants also release periplasmic proteins into the extracellular medium⁷⁹, but the amount of, for example, periplasmic glucose-binding protein that remains in the periplasm of the mutants is similar to that found in the wild-type strain⁸¹. Moreover, not only the transport systems that depend on a periplasmic binding protein are altered in these mutants, since glycerol transport, which is not dependent on periplasmic binding proteins, is also affected^{81, 115}.

Nevertheless, a conclusive reason for this transport defect is still unknown. The reduction in transport across the cytoplasmic membrane does not appear to be a consequence of the absence or reduced amounts of specific transport proteins in the cytoplasmic membrane, since the overexpression of the PutP inner membrane transporter does not enhance the uptake of proline in the *P. putida tol-oprL* mutants⁸¹. On the other hand, although the energy-dependent transport of glucose and arginine might have been influenced by the generation of pmf, the *tol-pal* mutants of *P. putida* are not particularly defective in the pmf generation, excluding the possibility that the reduced transport rates in the *tol-oprL* mutants are due to a low pmf at the cytoplasmic membrane⁸¹. Consistently, the transport of glycerol, which is taken up through a facilitated diffusion process that does not need energy, was also affected in the *P. putida tol-oprL* mutants, as well as in the *P. aeruginosa* and *E. coli tol-oprL(pal)* mutants.

The possibility remains that the Tol-OprL system could directly or indirectly influence the correct insertion or functioning of certain transport systems in the cytoplasmic membrane, but more detailed studies are still necessary to determine the underlying mechanisms that cause this phenotype.

5.3. Outer Membrane Biogenesis

5.3.1. Role in Porin Export and/or Assembly. The theory, still not proven, that the *E. coli* Tol–Pal system might have a role in the dynamic assembly of outer membrane components arose some years ago. In 1986, Lazzaroni and co-workers⁶⁹ reported that a *lkyB* (*tolA*) mutation in *E. coli* affected the expression of *ompF* and *ompC* genes at the transcriptional level (about 1.6-fold decrease). Similarly, the LamB protein content in the membrane of this mutant strain was shown to be reduced. Despite these modest changes, they suggested that the TolA protein could be necessary for correct integration or processing of these outer membrane proteins. Further *in vitro* experiments showing that TolA and TolB interacted with trimeric outer membrane proteins^{33, 112} supported the hypothesis that the *E. coli* Tol–Pal system could be involved in the process of translocation of outer membrane proteins through the periplasm and/or in their correct assembly into the membrane^{66, 70, 82}. Moreover, cell fractionation experiments demonstrated that the products of this gene cluster are preferentially associated with contact regions between the inner and outer membrane⁴⁵, which suggests that they might participate as a functional site of export of cell envelope components through the periplasm. However, the presence and correct assembly of porins in the outer membrane of *E. coli tol–pal* mutants⁶⁶ argues against this hypothesis. Moreover, *in vitro* porin assembly in the absence of the TolA, TolB, or Pal proteins is as efficient as it is in their presence²⁸. It has also been suggested²⁴ that the Tol–Pal(OprL) proteins might help high-molecular-weight molecules, such as porin trimer–lipopolysaccharide (LPS) complexes, to cross the peptidoglycan, which has an estimated cut-off value of 50 kDa for the passage of proteins³⁰.

In *P. putida*, immunodetection analyses indicate that the levels of OprD, OprB, and OprF proteins in the outer membrane of *tol–oprL* mutants are similar to those found in the wild-type strain⁸¹, suggesting that the Tol–OprL system is not involved in the export of these porins to the outer membrane of this microorganism. Moreover, Mills and Holloway⁸⁷ also did not find major differences in the cell envelope protein pattern of a *P. aeruginosa tol* mutant.

5.3.2. Role in LPS Export and/or Assembly. A recent study indicates that in *E. coli*, the TolA protein, but not TolQ, TolR, or TolB is required for surface expression of O-lipopolysaccharide⁴⁰. Based on the abnormal accumulation of mannose, a sugar unique to the O7 subunit in the cytoplasmic membrane of a *tolA* mutant, a role was proposed for TolA in the translocation of the O-antigen subunits across the inner membrane or in their subsequent assembly on the periplasmic side of the membrane. Furthermore, *E. coli lky* mutants (putative *tol* mutants) were shown to

possess a decreased neutral sugar content in their core LPS⁵. Whitfield and Valvano¹³² also reported the inability of some *E. coli tol* mutants to produce a smooth LPS. In *P. aeruginosa*, a putative *tol* mutant (arbitrarily designated as “*tolA*”) was proposed to have modifications in the core or lipid A region of the LPS¹¹³. The analysis of LPS content in *P. putida tol-oprL* mutants has not revealed, however, differences in the amount of O-antigen LPS compared with the wild-type, suggesting that in this bacteria, the Tol–OprL system is not involved in LPS biogenesis (M. Llamas, J. J. Rodríguez-Herva, and J. L. Ramos, unpublished results). This also seems to be the case in *S. enterica* sv. Typhimurium, where *tol* mutants display normal amounts of smooth LPS¹⁰⁶.

Since the Tol–OprL(Pal) system is well conserved among gram-negative bacteria, its function(s) is also expected to be conserved. Thus, it is probable that the aforementioned LPS phenotypes are an indirect effect of a global cell envelope disorganization.

5.4. Peptidoglycan Metabolism

The similarity in sequence and structure of the TolB C-terminal domain with the β -propeller domain of prolyl oligopeptidases generated the hypothesis that this protein might be involved in the recognition of certain peptidic structures^{1, 18}. In addition, a sequence similarity between the central channel of this domain and the active site of class B metallo- β -lactamases suggests that TolB might interact with the D-alanyl-D-alanine compound which is central to the biosynthesis and recycling of peptidoglycan and its ligation with lipoproteins¹. The TolB C-terminal domain seems to lack, however, the flexibility required to perform a catalytic function, which could be performed by the TolB N-terminal domain or another member of the Tol–Pal system¹. Abergel and co-workers¹ also suggest that TolB might directly interact with the peptidoglycan. Although the pathway of peptidoglycan biosynthesis is well known, this protein could be involved in pathways less well understood, such as those responsible for the recycling of peptidoglycan or the assembly of murein lipoproteins^{100, 134}. On the other hand, in *Chlamydia trachomatis* and *Chlamydia pneumoniae*, the gene downstream of *pal* (occupying a position equivalent to *ybgF*, and apparently belonging to the *tol-pal* gene cluster) shares similarity with the *amiA* gene of *E. coli*, a probable N-acetyl-muramoyl-L-alanine amidase¹¹⁹, suggesting, once more, a relationship between the Tol–Pal(OprL) system and cell wall metabolism. In any case, this hypothesis is based only on sequence predictions and currently lacks any experimental support.

6. CONCLUDING REMARKS

Despite the great number of studies published about the Tol system, especially in *E. coli*, the most important question: “what is the exact role of this protein complex?”, still remains unknown. Our knowledge of the protein–protein interactions that take place within this complex has improved substantially in the last years, nevertheless, the sincere statement made by B. W. Holloway and co-workers 30 years ago in their work describing the first *Pseudomonas tol* mutant⁵² could still be considered valid at this moment: “The phenotypic description of these *tol* mutants in fact raises more problems than solutions.” Actually, one of the most difficult problems in determining the actual function(s) of the Tol–OprL(Pal) system is how to distinguish between direct and indirect effects of the *tol* mutations.

The availability of the crystal structures of some components of the complex, many of them still in progress^{1–3, 18, 83, 133}, will undoubtedly be an important tool for understanding the physiological role of these proteins. On the other hand, it is quite clear that the Tol–OprL(Pal) system together with the Lpp and OmpA proteins (and probably with their respective equivalents, OprI and OprF, in *Pseudomonas*) constitute a structural network to link the outer membrane and the peptidoglycan. Thus, future research should also be focused on the analysis of this cell component. Bacteria must correctly synthesize and transport the cell wall components to maintain outer membrane stability, and also synchronize their integration while cells grow and divide. To understand the blebbing phenotype of the different *tol* mutants it will be interesting to analyse the peptidoglycan structure, especially in relation to its association with proteins, and measure its rate of synthesis in these mutants. In addition, the unexpected finding that certain inner membrane uptake systems appear to require an intact Tol–OprL(Pal) system to function correctly, should focus our attention not only on the outer membrane but also at the cytoplasmic membrane level. Remarkably, these defects, in contrast to those related with the LPS, are shared by *tol* mutants from all bacterial species analysed thus far, as one would expect from a mutant phenotype linked to the real function of the Tol–OprL(Pal) system. Finally, major efforts should be made to understand the role of the TolA–Pal interaction. Is it, by analogy to the Ton system, involved in transducing energy from the cytoplasmic to the outer membrane? If so, which specific event(s) does it control?

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EFFLUX PUMPS

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1. INTRODUCTION

Organisms such as *Pseudomonas aeruginosa*, *Stenotrophomonas maltophilia* and *Burkholderia* spp. are of increasing clinical importance because of their innate resistance to multiple agents and their ability to develop high-level multidrug resistance (MDR)^{52, 53}. This resistance owes much to the presence of broadly specific efflux systems which export and, thus, provide resistance to multiple antimicrobials. Drug efflux systems have been grouped into five families, the major facilitator (MF) family¹⁹⁶, the ATP-binding cassette (ABC) family^{101, 223}, the resistance-nodulation-division (RND) family^{222, 237}, the small multidrug resistance (SMR) family³¹ (a member of the much larger drug/metabolite transporter (DMT) superfamily⁸⁰) and the multidrug and toxic compound extrusion (MATE) family²² (see refs. [175, 183] for recent reviews on bacterial multidrug efflux systems). With the exception of the ABC exporters, which utilize ATP hydrolysis to promote drug extrusion, these cytoplasmic membrane-associated efflux pumps function as secondary transporters, coupling drug efflux to ion (H^+ or Na^+) influx. From a clinical standpoint, efflux systems of the RND family are most important, providing as they do efflux of and resistance to a variety of clinically relevant antibiotics and biocides^{173, 175}. Members of this family operate as part of a tripartite efflux system that also includes an outer membrane (the channel-forming OMF, outer membrane factor¹⁶⁵) and periplasmic (the membrane fusion protein, MFP³⁷) component

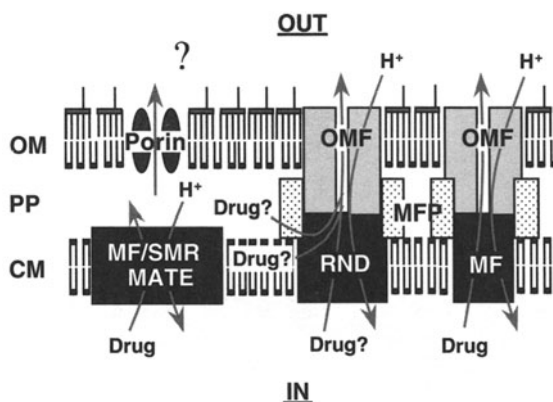


Figure 1. Schematic showing the organization and operation of multidrug efflux systems in the cell envelope of *P. aeruginosa*. RND type systems accommodate antimicrobials that act on periplasmic, cytoplasmic membrane and cytoplasmic targets and though it is not entirely clear where these agents are first 'captured' by the pump, current models suggest the periplasm or, perhaps, the outer leaflet of the cytoplasmic membrane. A description of the various efflux components is provided in the text. OM, outer membrane; PP, periplasm; CM, cytoplasmic membrane.

(reviewed in ref. [237]), an organization seen on occasion with MF transporters (Figure 1). Such tripartite efflux systems have been reported in *P. aeruginosa*, *S. maltophilia* and *Burkholderia* spp. where they contribute significantly to intrinsic and/or acquired resistance to a variety of antimicrobial compounds^{172, 174}. Related 3-component efflux systems are also present in non-pathogenic *Pseudomonas* spp., such as *Pseudomonas putida*, where they have been implicated in organic solvent tolerance rather than antimicrobial resistance^{172, 174, 185}.

2. *PSEUDOMONAS AERUGINOSA*

P. aeruginosa is an opportunistic human pathogen that is historically difficult to treat owing to an innate resistance to many antimicrobials and an ability to develop resistance during antimicrobial therapy⁷⁰. Indeed, a major predisposing factor for *P. aeruginosa* infection, at least in the hospital, is prior antibiotic usage (e.g., refs [13, 28, 41, 63, 211]). Long attributed to the outer membrane, a barrier of limited permeability¹⁵², the intrinsic multidrug resistance of this organism results from the synergy between outer membrane impermeability and chromosomally encoded multidrug efflux pumps^{54, 110}. Multidrug efflux systems of all families have either been described or homologues identified in *P. aeruginosa* (Table 1) though examples of the RND-MFP-OMF type are clearly the most important as regards resistance to clinically relevant antimicrobials^{172, 175, 180}.

Table 1. Multidrug efflux systems in *Pseudomonas* spp. and related organisms.

Organism	Efflux components ^a					Regulatory gene(s)	Substrates ^b	References ^c
	MFP	RND	OMF	SMR	MF	MATE		
<i>Burkholderia cepacia</i>	CeoA (AAB58160)	CeoB (AAB58161)	OpcM				CM,CP;TP (AAG21824) TC, NAL ML,AG	[23], [24], [25] [226] [139]
<i>Burkholderia pseudomallei</i>	AmrA	AmrB	OprA		BcrA			
<i>Burkholderia vietnamiensis</i>						Norm	PX	[46]
<i>Pseudomonas aeruginosa</i>	MexA	MexB	OprM				BL,FQ,CM,NV, TP,TG,SM,EB, AC,CV,SDS,AH, HL,CL,TL,IR,TS, TPPRD,LM,MU	[61], [178], [179], [181]
	MexC	MexD	OprJ				BL,FQ,CM, TC,NV,TP,ML, CV,EB,AC,SDS, AH,CL,TS,TG, LM,TPPRD,BA, CHX,MU	[177]
	MexE	MexF	OprN				FQ,CM,TP,AH,TS	[98]
	MexX (AmrA)	MexY (AmrB)	OprM [§]				BL,FQ,AG, TC,ER, TG,MU	[3], [138], [225]
	MexJ	MexK	OprM [§]				TS, ERY,	[30]
	MexH ⁱ	MexI ⁱ	OpmD ⁱ				TC, Cp ^h VA, HL?, EB, NOR, RD, AC	[2], [207a]

Table 1. Continued

Organism	Efflux components ^a					Regulatory gene(s)	Substrates ^b	References ^c
	MFP	RND	OMF	SMR	MF	MATE		
	PA1435 (AAG04824)	PA1436 (AAG04825)	?				?	
	PA4374 MexV	PA4375 MexW	OprM				FQ, CM, TC, [106a] ER, EB, AC	
	PA3523 (AAG06911)	PA3522 (AAG06910)	PA3521 ^j (AAG06909)				?	
	PA0156/ PA0157	PA0158 (AAG03548)	?				?	
	(AAG03546/ AAG03547)							
	PA2528 ^k (AAG05916)	PA2526/ PA2527 ^k (AAG05914/ AAG05915)	PA2525 ^{k,l} (AAG05913)				?	
				PA4990; a.k.a. EmrE (AAG08375)			?	EB, ACR, [107] MV, AG
				PA1540 ^m (AAG04929)			?	
				PA1541 ^m (AAG04930)			?	
				PA1882 ^m (AAG05271)			?	

<i>Pseudomonas putida</i>	PA5160; a.k.a EmrA ^P (AAG08544)	PA5158; a.k.a. OpmG (AAG08543)	PA5159; a.k.a. EmrB ^P (AAG08545)	PA5157 ^{jo} (AAG08542)	?	?	[103]
			PA4136 ^q (AAG07523)	?	?		
			PA3573 ^q (AAG06961)	?	?		
			PmpM _i ; ? a.k.a. NorM1	?	FQ, EB, AC, BA, TPP		[70a]
			PA5294; ? a.k.a. NorM2 ^r (AAG08679)	?	?		
	ArpA	ArpB	ArpC	<i>arpR</i>	TC,CM,CB, ST,ER,NV		[91]
	MepA	MepB	MepC	<i>mepR</i>	BL,TC,NV, ER,AH		[51]
	SepA	SepB	SepC	<i>sepR</i>	AH		[167]
	SrpA	SrpB	SrpC	<i>srpR</i>	AH		[92], [224]
				(AAFI6682)			
				<i>srpS</i>			
				(AAFI6681)			
	TtgA	TtgB	TtgC	<i>ttgR</i> , <i>ttgX^s</i>	CM,AP, TC,AH		[38], [186] [187], [218a]

Table 1. Continued

Organism	Efflux components ^a					Regulatory gene(s)	Substrates ^b	References ^c
	MFP	RND	OMF	SMR	MF	MATE		
<i>Stenotrophomonas maltophilia</i>	TtgD	TtgE	TtgF				TO, STY	[143], [185]
	TtgG	TtgH	Ttgi				AH,APCB	[193], [194]
	SmeA	SmeB	SmeC				BL,AG,FQ ^t	[111]
	SmeD	SmeE	SmeF				TC,ER,FQ,EB	[9], [198]

^aMultidrug efflux systems, known or predicted, of the three-component RND-MFP-OMF and MF-MFP-OMF types and the possibly single-component SMR, MF and MATE types are indicated. In instances where a putative efflux system has been identified simply by homology (e.g., using a BlastP search of the *P. aeruginosa* genome sequence with representatives of the various efflux pump components/types) and no experimental evidence exists in support of a multidrug export function, the 'PA' designations (and gene names where given) provided by the *Pseudomonas* Genome Project (<http://www.pseudomonas.com>) and the Genbank protein accession numbers (in parentheses) are highlighted. ^bAC, acriflavine; ACR, acridines; AG, aminoglycosides; AH, aromatic hydrocarbons; AP, ampicillin; AZ, azithromycin; BA, benzalkonium; BL, β -lactams; CB, carbenicillin; CHX, chlorhexidine; CL, cerulenin; CM, chloramphenicol; CP, ciprofloxacin; CV, crystal violet; EB, ethidium bromide; ER, erythromycin; FQ, fluoroquinolones; GN, gentamicin; HL, homoserine lactones; IR, irgasan; LM, lincomycin; ML, macrolides; MU, mureidomycin A and C; NAL, nalidixic acid; NOR, norfloxacin; NV, novobiocin; PX, polymyxin; RD, rhodamine; SDS, sodium dodecyl sulphate; SM, sulphonamides; ST, streptomycin; STY, styrene; TC, tetracycline; TG, tigecycline; TL, thiolactomycin; TO, toluene; TS, triclosan; TP, trimethoprim; TPP, tetraphenyl phosphonium; VA, vanadium. In instances where only one member of a class of antimicrobial has been tested or is known to be a substrate for a given pump, that member is identified. Where several members of an antimicrobial class are known to be substrates, the class is identified rather than the actual compounds tested.

^cReferences related to the efflux genes and their regulators only are provided. Details of substrate specificity are cited in the text.

^d*ceoR* occurs upstream of *ceoAB-opcM* and encodes a product showing homology to the LysR family of regulators. Still, its involvement, if any, in the regulation of *ceoAB-opcM* expression remains to be elucidated.

- ^cThe *nalC* gene (a.k.a. PA3721 as designated by the *Pseudomonas* Genome Project [<http://www.pseudomonas.com>]) is unlinked to the *mexAB-oprM* genes but encodes a TetR/AcrR family repressor whose inactivation leads to *mexAB-oprM* hyperexpression (Cao, Srikumar and Poole, unpublished).
- ^dMexT is required for MexEF-OprN expression in *nxkC* mutants though the nature of the mutation(s) responsible for hyperexpression of the efflux genes in such mutants is unclear.
- ^eIn light of the demonstration that null mutations in the OMF homologues *opmG*, *opmI* and *opmH* compromise aminoglycoside resistance in wild type cells of *P. aeruginosa*⁸⁵ it is unclear whether OpmI is the only or even the intended OMF for the MexXY efflux system.
- ^fEfflux of triclosan but not the other antimicrobials requires MexJK without OpmI involvement. Recent evidence indicates that MexJK work with the OMF OpmH, the probable *P. aeruginosa* TolC homologue, in the export of and resistance to triclosan (H. Schweizer, personal communication).
- ^gEncoded by a putative four-gene operon, *mexGHJ-opmD* operon, although the significance/function of the MexG component is unclear².
- ^ha.k.a. OpmE¹⁴⁵.
- ⁱThe PA2528-PA2527-PA2526 gene products show substantial similarity to the YegMNO¹⁵⁴ (a.k.a. MdtABC^{18, 147}) efflux components of *E. coli* (<http://www.pseudomonas.com/>).
- ^ja.k.a. OpmB¹⁴⁵.
- ^mThese EmrE homologues of the SMR family were identified (though not characterized) by Li *et al.*¹⁰⁷.
- ⁿThis defective version of the QacE multidrug exporter of the SMR family¹⁶⁴, was reported in a single *P. aeruginosa* isolate, although no correlation with resistance to intercalating dyes and quaternary ammonium compounds, the normal substrates for this exporter, was seen.
- ^oThis first gene of the four-gene operon PA5157-PA5158-PA5159-PA5160 encodes a putative repressor of the MarR family (<http://www.pseudomonas.com/>) that includes the EmrR repressor of the *emrAB* operon of *E. coli*¹¹⁹.
- ^pThe PA5159-PA5160 gene products (also called PmrAB; see Genbank accession numbers AAC31387 and AAC31388) show substantial similarity (44–48% identity) to the *E. coli* EmrAB multidrug efflux components¹¹⁸ and likely function as a three-component MF-MFP-OMF efflux system with PA5158 as the outer membrane OMF component.
- ^qTwo of the many MF type transporters identifiable in the *P. aeruginosa* genome sequence (<http://www.pseudomonas.com/>), these show the highest similarity (c. 25% identity) to the MdtA multidrug transporter of *E. coli* that has been shown to accommodate a broad range of antimicrobials^{20, 40}.
- ^rDemonstrates significant amino acid sequence homology to the NorM multidrug transporter first identified in *V. parahaemolyticus* and *E. coli* (a.k.a. YdhE in this latter organism)¹⁴⁰.
- ^sAlthough disruption of a *lrp*-like *tigX* gene was reported to enhance *tigR* expression and decrease expression of both *tigABC* and *tigDEF*, implicating this protein as a putative global regulator of efflux systems in *P. putida*³⁸, this gene is now known to play no role in efflux gene regulation¹⁸⁵.
- ^tAntimicrobial resistance is independent of SmeAB but requires SmeC, probably in conjunction with other, as yet unidentified RND-MFP components.

2.1. RND-Type Efflux Systems

To date, 5 RND-MFP-OMF type multidrug efflux systems have been described in *P. aeruginosa*, including MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM and MexJK-OprM (Table 1; reviewed in refs [172], [176], [180]), with OprM functioning as the OMF component of three of these. This latter is reminiscent of the *Escherichia coli* TolC protein, which functions as the OMF for several MDR efflux systems in this organism as well (reviewed in refs [173, 175]). A sixth RND-MFP-OMF type efflux system has recently been described in *P. aeruginosa*². Dubbed MexGHI-OpmD, this pump provides resistance to vanadium and impacts quorum-sensing-dependent processes (loss of the pump compromises expression of several products known to be regulated by quorum-sensing including N-acyl homoserine lactones [AHL]) suggestive of a role in AHL homeostasis, though no impact on antimicrobial resistance was reported². While these efflux systems do contribute to antimicrobial resistance, it is important to note that they often function synergistically with additional resistance mechanisms in promoting resistance (e.g., refs [73], [117], [130], [150], [217]).

2.1.1. Substrate Specificity. The MFP-RND-OMF type multidrug efflux systems of *P. aeruginosa* exhibit a very broad substrate specificity, accommodating as they do a variety of antibiotics as well as biocides, organic solvents, dyes, detergents, metabolic and energy inhibitors, homoserine lactones and, possibly, virulence factors^{172, 175, 180}. The ability of these systems to accommodate both biocides (i.e., antiseptics and disinfectants) and antibiotics is of particular concern given the possible selection of multiple antibiotic resistant strains by biocides such as triclosan whose use in the community at large is increasing²⁰⁶. Numerous studies implicate the RND components in substrate recognition by these tripartite multidrug efflux systems^{39, 126, 146, 220} although the nature of this broad substrate specificity (i.e., recognition of multiple, structurally unrelated substrates by a single efflux protein) is as yet poorly understood. Recent mutagenesis studies on the MexD¹²⁶ and MexB^{136a} RND components as well as information from crystal structures of the drug-bound forms of multidrug-binding transcriptional regulators that control expression of multidrug efflux systems in Gram-positive organisms^{57, 204} may, however, provide some insights.

2.1.1.1. MexAB-OprM. The first *P. aeruginosa* multidrug efflux system to be described, MexAB-OprM^{61, 108, 178, 179} is expressed constitutively in cells grown in standard laboratory media, where it contributes to intrinsic resistance to a number of antimicrobials including fluoroquinolones, tetracyclines, macrolides,

chloramphenicol, novobiocin, trimethoprim, sulphonamides and lincomycin^{96, 136}. This efflux system also exports and contributes to resistance to the biocide triclosan²⁰⁵, the recently described glycyclcycline tigecycline (a.k.a. GAR-936)³⁵, the novel peptidoglycan-inhibiting peptidynucleotide antibiotic mureidomycin A/C⁵⁹ as well as β -lactams^{136, 214} (including β -lactamase inhibitors¹¹²). Of the β -lactams, the MexAB-OprM pump accommodates a variety of penicillins and cepheids, including such agents as carbenicillin and aztreonam, which are uniquely exported by this system¹³⁶. It also provides modest resistance to some carbapenems (but not imipenem)¹⁵⁸ and is a major reason for the noted high-level intrinsic resistance of *P. aeruginosa* to penems^{157, 159}. The overall significance of MexAB-OprM vis-à-vis β -lactam resistance does, however, vary with the β -lactam examined, in some instances playing a greater or lesser role than, for example, the chromosomally encoded β -lactamase of *P. aeruginosa*^{130, 148, 217}. MexAB-OprM also contributes to the net resistance of penicillin-binding protein mutants, inasmuch as loss of this efflux systems compromises, to some extent, β -lactam resistance in such mutants²¹⁷.

MexAB-OprM is also hyperexpressed in *in vitro*-selected^{96, 132, 133, 181, 188} and clinical^{126, 73, 77, 82, 83, 168, 244} *nalB* (a.k.a. *cfxB*²⁷) mutants, which are, thus, resistant to multiple antibiotics. Although such resistance was typically seen in planktonic cells and suggestions were made that MexAB-OprM may also be important for the resistance of biofilm-grown *P. aeruginosa* to certain antimicrobials²¹ though this appears not to be the case³⁴. Though often selected by fluoroquinolones *in vitro*⁹⁹ and *in vivo*⁸⁶, strains hyperexpressing MexAB-OprM have also been identified amongst *in vitro*-isolated tetracycline-^{7, 68, 84} and chloramphenicol-resistant strains⁸⁴, and in clinical isolates resistant to β -lactams²⁶, including the carbapenem meropenem¹⁶².

In addition to medically relevant antimicrobials, MexAB-OprM also exports a variety of dyes^{54, 107, 155, 216} and detergents²¹⁴, biocides (e.g., the fatty acid biosynthesis inhibitor triclosan)²⁰⁵, organic solvents (a.k.a. aromatic hydrocarbons)^{109, 114} and homoserine lactones associated with quorum sensing^{44, 166}. The latter play a role in cell density-dependent expression of a number of virulence factors in *P. aeruginosa* and, thus, the activity of this efflux system can influence virulence²⁰⁰. Indeed, a recent study suggests that the MexAB-OprM efflux system of *P. aeruginosa* promotes the release of molecule(s) ultimately important for the virulence of this organism⁷⁵. The observation that MexAB-OprM hyperexpression in *nalB* strains impairs fitness and virulence²⁰⁰ also suggests that this efflux system has a physiological role in *P. aeruginosa* independent of antimicrobial efflux and resistance. Consistent with this, mutants hyperexpressing MexAB-OprM were readily selected *in vivo* in a rat model of acute *P. aeruginosa* pneumonia in the absence of any antibiotic selection⁸⁶. The specific nature of the selective *in vivo* growth advantage provided by this efflux system is, however, unknown.

2.1.1.2. MexCD-OprJ. The MexCD-OprJ multidrug efflux system¹⁷⁷ is not detectable in wild type cells, at least under usual laboratory growth conditions^{78, 214} though it is inducible by a number of non-antibiotic substrates including tetraphenylphosphonium chloride, ethidium bromide, rhodamine, acriflavine and the biocides benzalkonium chloride and chlorhexidine^{141, 142}. These agents, thus, render the organism resistant to several antibiotics known to be substrates for this efflux system (see below)^{141, 142}. Given the routine use of benzalkonium and chlorhexidine as antiseptics in, for example, hospitals, the possible up regulation of MexCD-OprJ and, thus, induction of multidrug resistance may be of concern. Expression of this efflux system is also seen in *nfxB* MDR mutant strains isolated *in vitro*^{74, 133, 177} and in the clinic^{81, 82, 83, 233}. Originally identified as a determinant of fluoroquinolone resistance⁷⁴, MexCD-OprJ accommodates a variety of antimicrobial agents including macrolides, chloramphenicol, novobiocin, tetracycline, trimethoprim, tigecycline, mureidomycin A/C and a variety of β -lactams^{35, 59, 62, 131, 136, 214}. Although the MexCD-OprJ export of β -lactams was originally reported to be limited to fourth generation cepheems (e.g., cefpirome and cefepime)^{131, 177}, more recent studies using mutants lacking MexAB-OprM but expressing MexCD-OprJ have confirmed the ability of this efflux system to accommodate and, thus, provide resistance to a variety of ordinary cepheems^{62, 136} as well as several penicillins¹³⁶. Like MexAB-OprM, this efflux system also provides modest resistance to some carbapenems (but not imipenem)¹⁵⁸ and penems¹⁵⁹, though in the latter case only in the absence of MexAB-OprM. This efflux system also exports the biocide triclosan, which readily selects for MexCD-OprJ-hyperexpressing *nfxB* strains *in vitro*²⁹. Export of a variety of non-antimicrobials has also been reported, including dyes, detergents and organic solvents^{109, 213, 214}. As with *nalB* mutants, *nfxB* mutants show reduced fitness and virulence²⁰⁰.

An intriguing feature of *nfxB* strains is their hypersusceptibility to β -lactams such as carbenicillin^{62, 74, 214}, which appears to result from the reduced expression of MexAB-OprM⁶² and the AmpC β -lactamase¹³⁴ in these mutants. The observation that *nfxB* mutants are also hypersusceptible to aminoglycosides^{74, 177}, a major substrate for the MexXY-OprM multidrug efflux system (see below), suggests that this efflux system, too, may be down regulated in *nfxB* mutants. The coordinated expression of MDR efflux systems in *P. aeruginosa*, with increases in one compensated for by decreases in another also extends to additional multidrug efflux systems in this organism¹¹³, though the significance of this is, at present, unclear.

2.1.1.3. MexEF-OprN. The MexEF-OprN system is apparently quiescent in wild type cells, at least under the usual laboratory growth conditions⁹⁸, but is expressed in *nfxC* type multidrug resistant strains isolated *in vitro*^{49, 98, 133} and

in the clinic^{50, 82}. Generally selected as fluoroquinolone-resistant mutants^{49, 99}, *nfxC* mutants have also been isolated on media containing tetracycline or chloramphenicol^{82, 98}. *nfxC* mutants display resistance to fluoroquinolones, chloramphenicol, trimethoprim and the carbapenem imipenem^{49, 98}. Although triclosan selection of a MexEF-OprN-hyperexpressing mutant has not been reported, this efflux system does accommodate triclosan and provides resistance to this biocide in the absence of MexAB-OprM²⁹. The observed hypersusceptibility of *nfxC* mutants to β -lactams and aminoglycosides⁴⁹, a phenotype shared with *nfxB* strains (see above), may result from decreased expression of MexAB-OprM and MexXY-OprM in these mutants. Intriguingly, resistance to imipenem in *nfxC* strains results not from MexEF-OprN expression⁹⁸ but the concomitant decrease in outer membrane protein OprD in these mutants^{49, 133}. OprD is an imipenem channel and a primary route of entry of this antibiotic in *P. aeruginosa*²²¹ whose absence is often seen in imipenem-resistant strains of *P. aeruginosa*^{16, 97}. Indeed, loss of OprD is the primary determinant of carbapenem resistance in clinical strains of *P. aeruginosa*¹⁶². In addition to its role in export of and resistance to antimicrobials, MexEF-OprN also promotes resistance (tolerance) to organic solvents¹⁰⁹, dyes⁵⁴ and biocides such as triclosan²⁹. As a result of MexEF-OprN hyperexpression, *nfxC* mutants also express reduced levels of several quorum-sensing (i.e., homoserine lactone)-dependent extracellular virulence factors¹⁰⁰ and are attenuated for virulence³², apparently as a result of MexEF-OprN-mediated export of molecule(s) necessary for homoserine lactone production¹⁰⁰.

2.1.1.4. MexXY-OprM. Unlike the aforementioned efflux operons, the *mexXY* system¹³⁸ (also called *amrAB*²²⁵) lacks a linked OMF gene¹³⁸, reminiscent of the *acrAB* MDR efflux operon of *E. coli* whose OMF gene, *tolC*, is also located elsewhere on the chromosome^{48, 123, 124}. MexXY apparently utilizes OprM as its outer membrane constituent^{3, 138}, although the recent demonstration that mutants lacking one of outer membrane proteins OpmG, OpmH or OpmI were aminoglycoside hypersusceptible suggests that one or more of these may also function with MexXY, perhaps as the intended OMF for this efflux system⁸⁵.

Strains deleted for *mexXY* show increased susceptibility to aminoglycosides, erythromycin, several tetracyclines and a variety of glycylicyclines, including tigecycline^{3, 35}, indicating that this efflux system contributes to the intrinsic resistance of *P. aeruginosa* to these agents. While the cloned genes also promote resistance to fluoroquinolones^{3, 138}, indicating that fluoroquinolones are accommodated by MexXY-OprM, this efflux system does not contribute to intrinsic resistance to these agents. Expression of *mexXY* is inducible in wild type *P. aeruginosa* by aminoglycosides, tetracycline, erythromycin and tigecycline but not by fluoroquinolones^{35, 135}, explaining the

lack of contribution of this system to intrinsic fluoroquinolone resistance. A recent paper also highlights the contribution of MexXY to resistance to macrolides, tetracyclines, lincomycin, chloramphenicol, novobiocin as well as several β -lactams, though, again, only in a mutant lacking MexAB-OprM¹³⁶. Of the β -lactams, MexXY-OprM accommodates several penicillins and cepheems¹³⁶ and provides modest resistance to a limited number of carbapenems (not imipenem)¹⁵⁸. Still, loss of *mexXY* from wild type cells does not impact, for example, β -lactam susceptibility, and β -lactams do not induce *mexXY* expression in wild type cells, indicating that this efflux system is not a determinant of intrinsic β -lactam resistance¹³⁶. Again, however, mutants lacking MexAB-OprM show β -lactam induction of *mexXY*¹³⁶. It appears, likely, then, that all known substrates of MexXY are capable of pump induction, dependent, however, on sufficient drug accumulating inside cells as can occur only when such accumulation is not thwarted by, for example, the activity of the MexAB-OprM efflux system. Since aminoglycosides¹⁰⁷, tetracycline^{135, 180} and erythromycin^{136, 214} are poor substrates for MexAB-OprM, these agents are clearly able to induce expression of the MexXY system even when MexAB-OprM is present.

The aminoglycoside inducibility of *mexXY* likely explains the phenomenon of adaptive aminoglycoside resistance (e.g., refs [88, 230]) displayed by recalcitrant subpopulations of *P. aeruginosa* that appear during aminoglycoside exposure⁷⁶. The well-known divalent cation antagonism of aminoglycosides in *P. aeruginosa* is also dependent on the presence of this efflux system¹²⁷. Finally, hyperexpression of MexXY/AmrAB is also seen in a number of so-called impermeability type aminoglycoside-resistant clinical strains of *P. aeruginosa*^{210, 225}, and elimination of this efflux system compromises this resistance in many instances, confirming the role of AmrAB/MexXY in the aminoglycoside resistance of these isolates^{210, 225}. An *in vitro* isolated mutant selected on gentamicin and ofloxacin also showed increased MexXY expression relative to wild type, though this was assessed following antibiotic induction of the efflux system¹³⁶. It is not clear, then, whether the MexXY hyperexpression was antibiotic-dependent or whether increased expression of *mexXY* relative to wild type would have been observed even in the absence of the inducer (as seen, e.g., in an *amrR/mexZ* mutant; see below).

2.1.1.5. MexJK-OprM. Triclosan-resistant derivatives of a Δ *mexAB-oprM*/ Δ *mexCD-oprJ* double mutant of *P. aeruginosa* hyperexpress yet a fifth efflux system of the RND type, encoded by the *mexJK* genes, that also provides modest resistance to antibiotics, though only in strains that express OprM³⁰. Thus, the *mexJK*-encoded MFP-RND components function with OprM as the OMF component in providing resistance to the antibiotics tetracycline and erythromycin³⁰. This represents the third known efflux system that

utilizes this OMF, and given the presence of additional 2-gene MFP-RND operons in the genome sequence (Table 1) this OMF may function as the outer membrane component of yet more efflux systems. Interestingly, MexJK-dependent triclosan resistance is independent of OprM³⁰ and appears to require yet another OMF, the *P. aeruginosa* TolC homologue, OpmH (H.P. Schweizer, personal communication). Still, given the apparent flexibility of OMF usage in *P. aeruginosa*, where, for example, OprM has been shown to replace both the OprJ component of the MexCD-OprJ pump (and vice versa)^{60, 214} and the OprN component of the MexEF-OprN pump¹²⁹, where the OpmB (a.k.a. PA2525) OMF component of a putative PA2528-2527/2526-2525 MFP-RND-OMF efflux system (Table 1) can replace OprM, OprJ and OprN¹⁴⁵, and where *E. coli* TolC can replace OprJ²¹³, it is unclear whether the ability of OprM to work with MFP-RND components other than MexAB is physiologically relevant.

2.1.1.6. MexGHI-OpmD. The most recently described efflux system of the RND family is encoded by an apparently 4-gene operon, *mexGHI-opmD*, although the MexG component shows no noteworthy homology to existing proteins and may be dispensable for function². This efflux system was first identified as a determinant of vanadate resistance and its ability to export antimicrobials has only recently been confirmed^{207a}.

2.1.1.7. Others. PA4374-PA4375 are now known to encode an efflux system, MexVW, that operates with OprM in facilitating resistance to several antimicrobials in *P. aeruginosa*^{106a}. The PA2528-PA2527-PA2526 gene products show substantial similarity to the YegMNO¹⁵⁴ (a.k.a. MdtABC^{18, 147}) efflux components of *E. coli* (<http://www.pseudomonas.com/>). Like the YegMNO/MdtABC system the putative PA2528-PA2527-PA2526 efflux system includes 2 RND components, which, given the recently revealed trimeric form of an RND pump (AcrB) in *E. coli*¹⁴⁴ may form heterotrimers.

2.1.2. Pump Architecture. The typical model of an RND-MFP-OMF pump describes the cytoplasmic membrane drug-proton antiporter (RND) and the outer membrane-spanning OMF as interacting in the periplasm, such interaction possibly promoted and/or stabilized by the periplasmic (but attached to the cytoplasmic membrane by an N-terminal lipid tail) MFP component that interacts with both¹⁷⁰ (Figure 1). Crystal structures are not available for the pump components of any of the *P. aeruginosa* efflux systems although the OMF (i.e., TolC) and RND (i.e., AcrB) components of the highly homologous (to the Mex systems) AcrAB-TolC multidrug efflux system of *E. coli* have been crystallized and offer insights into the organization and operation of this family of multidrug transporters^{102, 144}.

The TolC channel is a novel structure, existing as a trimer and spanning both the outer membrane (as a β -barrel composed of 12 membrane-spanning segments, four from each monomer) and periplasm (as a α -helical barrel)¹⁰². Measuring 140 Å in length, the channel is open at the distal (extracellular) end and tapers almost to a close at the proximal (periplasmic) end that likely interacts with the RND component, AcrB¹⁰². Modeling studies of the OprM OMF component of the MexAB-OprM, MexXY-OprM and MexJK-OprM multidrug efflux systems of *P. aeruginosa* (Table 1) indicate that this OMF adopts the same structure^{115, 227} (Figure 2) although unlike TolC⁶⁷ OprM is a lipoprotein and acylated at an N-terminal cysteine residue^{149, 179}. Still, this acylation appears to be dispensable for OprM function^{115, 149}. Studies with the purified TolC¹⁹ and OprM²²⁸ proteins confirm them as forming very small channels, in agreement with the proximal tapering of these channels (Figure 2) and

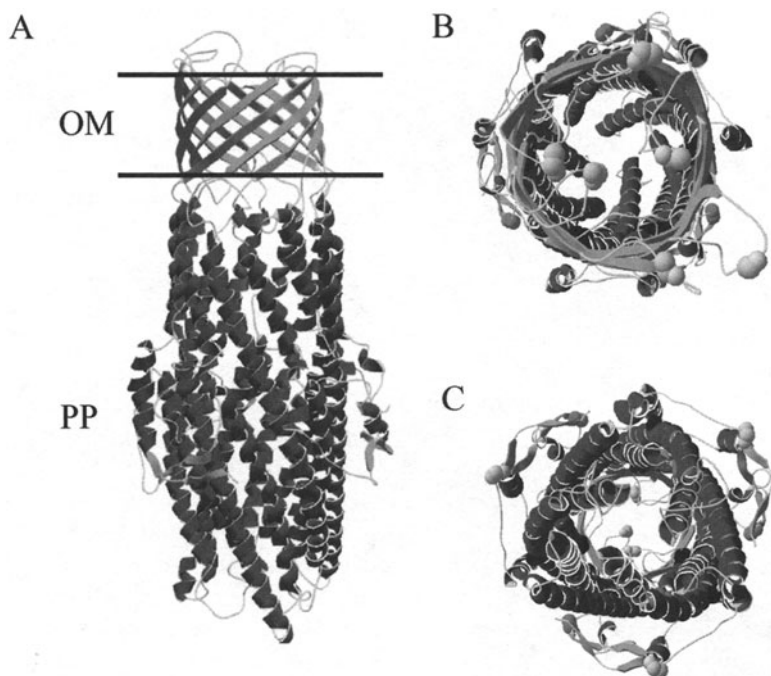


Figure 2. Three-dimensional model of OprM obtained by threading the amino acid sequence of OprM on the TolC crystal structure. Side (A), end-on (top) (B) and end-on (bottom) (C) views of the OprM trimer are shown. The probable outer membrane (OM) and periplasmic (PP) portions of the protein are highlighted. Note that the opening at the outer membrane surface (B) tapers almost to a close at the proximal end of the protein in the periplasm (C). Taken from ref. [227] with the permission of the authors and the American Society for Microbiology.

consistent with a need for a conformational change to promote opening of these probably 'gated' channels to enable them to accommodate drugs and other substrate molecules^{11, 234}. Such gating has recently been suggested for OpmM²³⁴ and may be mediated by the TonB1 energy coupler implicated in the 'gating' of outer membrane receptors involved in iron-siderophore transport across the outer membrane¹⁸², inasmuch as *tonB1* mutants are multidrug hypersusceptible and appear to be deficient in multidrug efflux^{242, 243}. Intriguingly, a *tonB* mutant of *P. putida* also exhibits a multidrug hypersusceptible phenotype that appears to be explained by an efflux defect⁵⁵, and inducible resistance to hydrophobic agents mediated by the MtrCDE multidrug efflux system of *Neisseria gonorrhoeae* also requires the TonB protein of this organism¹⁹⁵.

The AcrB RND component also exists as a trimer that adopts a 'jellyfish-like' shape with a 50-Å thick transmembrane region and a 70-Å headpiece that protrudes into the periplasm¹⁴⁴. This headpiece has a funnel-like opening at the top (where it likely interacts with TolC) that is connected (via a pore) to a central cavity at the bottom that then opens to the periplasm via three vestibules present at the interface between adjacent monomers¹⁴⁴. These latter likely play a role in substrate recognition by and/or access to the AcrAB-TolC pump¹⁴⁴. Indeed, several recent studies highlight the role played by the periplasmic portion of the RND transporters of *E. coli* and *P. aeruginosa* in substrate (i.e., drug) recognition^{39, 42, 126, 146, 220, 234a}. MexB is highly homologous to AcrB and like AcrB it spans the cytoplasmic membrane 12 times with two very pronounced periplasmic loops between helices 1 and 2, and helices 7 and 8⁶⁵ (a topology also presented by MexD⁵⁸). Recent modeling of the MexB protein indicates that it adopts the same trimeric jellyfish structure (Figure 3). Mutations in MexB^{136a, 232} MexD¹²⁶ and MexF⁴ that alter the substrate specificity of the corresponding efflux systems have been reported and may, in light of the available 3D models for RND transporters¹⁴⁴ (Figure 3), shed light on the nature of multisubstrate recognition by these multidrug transporters. Preliminary mutagenesis studies have also confirmed the functional importance of a number of charged residues in transmembrane segments of the MexB⁶⁶ and MexF⁴ RND components of multidrug efflux in *P. aeruginosa*, including a pair of adjacent and essential aspartate residues in transmembrane segment 4 that are generally conserved in this family of efflux transporters and implicated in proton translocation²³⁷.

Although a high-resolution crystal structure for an MFP efflux component is not yet available, preliminary studies indicate that, for example, AcrA is also likely trimeric²³⁶ and that monomer AcrA is a highly asymmetric, elongated molecule of sufficient length to span the periplasm²³⁵. Indeed, a preliminary, low-resolution structure of AcrA confirms the elongated nature of protein, which displays a probable length of 210 Å¹⁵. Despite the suggestion

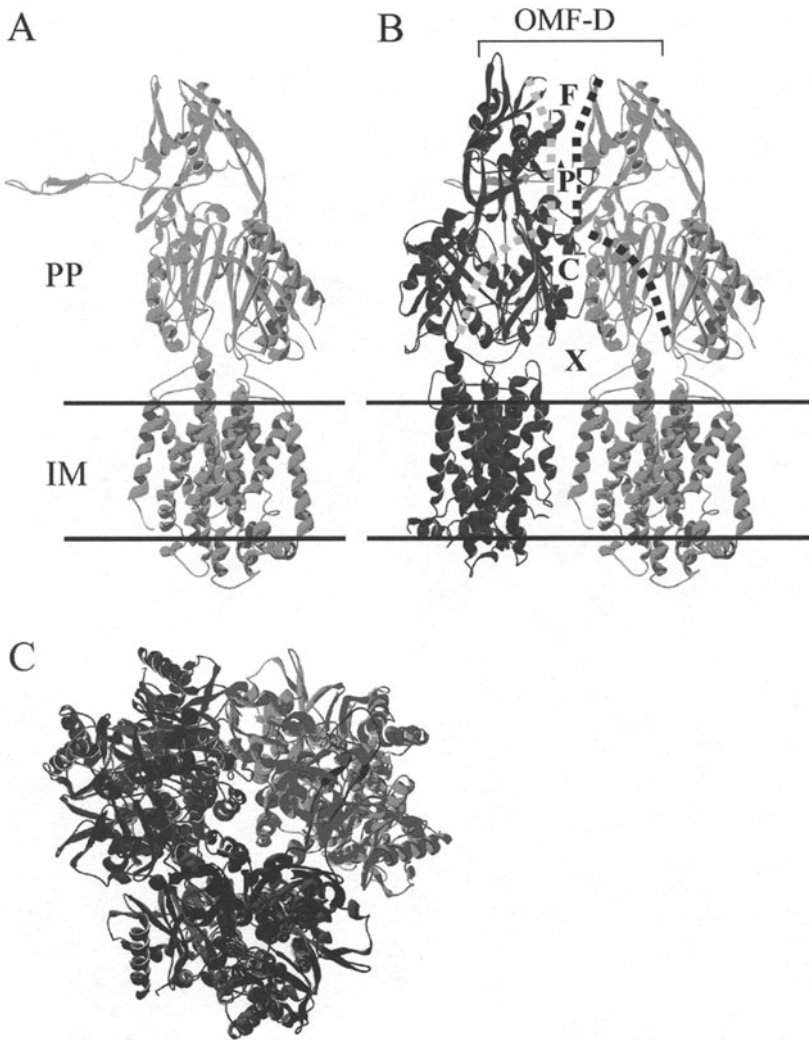


Figure 3. Three-dimensional structure of MexB obtained by threading the amino acid sequence of MexB on the AcrB crystal structure. (A) Side view of the MexB monomer. (B) Side (cut-away) view of the MexB trimer with the monomer closest to the viewer removed in order to show the vestibule (x) between the two remaining monomers. The probable inner membrane (IM) and periplasmic (PP) portions of the protein are highlighted. Dashed lines outline the central cavity (C), pore (P) and funnel (F) that exits the protein at the probable OMF (i.e., OprM)-docking domain (OMF-D) of MexB. C. End-on (top) view of the MexB trimer showing the pore/channel present in the periplasmic portion of the MexB trimer. Provided by G.A. McKay with the assistance of L. Loschi.

of OMF–RND component interactions in MFP-RND-OMF type efflux systems that is provided by the TolC and AcrB structures, an interaction between AcrB (or AcrA) and TolC has yet to be demonstrated²³⁶. Still, interactions between AcrA and AcrB^{89, 236} (and MexA and MexB [D. Nehme, X.-Z. Li, R. Elliot, and K. Poole, unpublished data]) have been confirmed and occur in the absence of substrate or the corresponding OMF component. This is reminiscent of the *E. coli* HlyBD–TolC hemolysin exporter, where the cytoplasmic membrane-associated Hly components also interact independent of substrate and TolC, with TolC being recruited by the Hly proteins only when substrate (i.e., hemolysin) enters the export pathway²¹⁹. Perhaps, then, TolC also associates with AcrAB only transiently when substrate is being exported. Given that TolC functions as the OMF of several export systems this would make sense. Similarly, the observation that OprM is the OMF of several multidrug efflux systems in *P. aeruginosa* (though see above) may explain the lack of a ‘constitutive’ association with its efflux counterparts as well. Like AcrA²³⁵, MexA is a lipoprotein²³¹, although acylation of these, like OprM, appears to be dispensable for function^{231, 235}.

2.1.3. Regulation. The genes encoding tripartite RND type efflux systems in *P. aeruginosa* (Figure 4) and other Gram-negative bacteria⁶⁴, including *B. pseudomallei*, *S. maltophilia* and *P. putida* (see below and Table 1) typically occur immediately adjacent to a gene or genes encoding regulatory (generally repressor) proteins that control efflux gene expression. In most instances, too, expression of these efflux genes in both lab and clinical multidrug resistant isolates occurs as a result of mutations in these regulatory genes, though some systems are clearly ‘drug’ inducible (e.g., MexCD–OprJ and MexXY; see above) while others are expressed constitutively at modest levels (e.g., MexAB–OprM; see above).

2.1.3.1. MexAB–OprM. A gene, *mexR*, occurs upstream of the efflux genes¹⁸¹ and is the target of mutations in *nalB* strains^{83, 181, 197, 215, 244}. A repressor²¹⁵, MexR, negatively regulates expression of *mexAB–oprM*^{181, 215} and is responsible for negative autoregulation of *mexR*¹⁸¹. MexR is a member of the MarR family of regulators¹³⁷. MarR, the product of the first gene of the *marRAB* operon (also called the *mar* locus) in *E. coli*, negatively regulates *marRAB* expression, thereby controlling expression of the transcriptional activator MarA and, thus, several MarA-regulated genes associated with resistance including the *acrAB* multidrug efflux operon⁵. The purified MexR protein binds as an apparent dimer to two sites in the *mexR–mexA* intragenic region⁴³, near *mexR* and overlapping promoters for *mexR* and *mexAB–oprM*^{43, 201}, thus explaining its negative control of *mexR* and *mexAB–oprM* expression. While most *mexR* mutations in *nalB* strains abrogate stable MexR

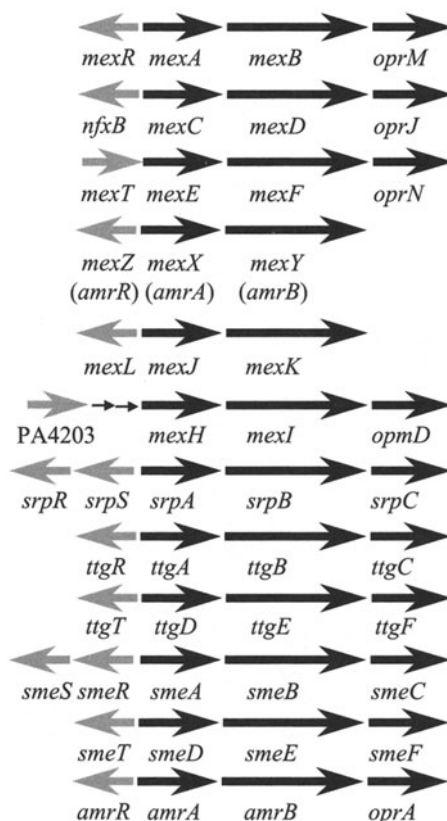


Figure 4. Presence of local regulatory genes (grey arrows) upstream of efflux genes (black arrows) of the MFP-RND-OMF family. With the exception of *mexT* and PA4203, which encode known (*mexT*) and putative (PA4203) positive regulators of the LysR family²⁰², and *smeRS*, which encodes a probable response regulator and sensor kinase, respectively, all of the regulatory genes encode known or probable repressors of the MarR²¹⁸ (*mexR*), LacI-GalR¹⁵¹ (*nfxB*), TetR¹² (*mexZ*, *mexL*, *ttgR*, *smeT*, *amrR*) and IclR²⁴¹ (*ttgT*, *srpS*) families. The *srpR* gene product shows modest similarity to AcrR, a TetR family negative regulator of the *acrAB* multidrug efflux genes of *E. coli*¹²². Because of the probable identity of the *mepR-mepoABC*-, *arpR-arpABC*- and *ttgR-ttgABC*-encoded systems, only the latter is depicted here. Similarly, because *sepR-sepABC* and *ttgT-ttgDEF* also likely encode the same efflux system, as do *srpRS-srpABC* and *ttgVW-ttgGHI*, only the first-described *ttgDEF* and *srpABC* loci are shown.

production (and, thus, MexR repressor activity), mutations that yield wild type levels of MexR have been described that appear to compromise MexR dimerization or DNA (i.e., operator) binding¹. A recent crystallographic study confirms that MexR is a dimer of the winged helix family¹¹⁶ that includes MarR⁶ as well as the BmrR⁷¹ and Mtn⁵⁶ regulators of multidrug efflux systems in *Bacillus subtilis*.

MexAB-OprM hyperexpression also occurs independently of mutations in *mexR* or the *mexR* and *mexAB-oprM* promoter regions^{215, 244}. These so-called *nalC* mutants²¹⁵ carry a mutation in a recently identified gene (PA3721, a.k.a. *nalC*) that encodes a TetR family¹² repressor of an adjacent probable 2-gene operon, PA3720-PA3719 (L. Cao, R. Srikumar and K. Poole, unpublished data). Apparently, it is the increased expression of PA3720-3719 (whose products show no significant homology to anything currently in the Genbank databases) that results from disruption of the *nalC* repressor gene that directly or indirectly promotes *mexAB-oprM* hyperexpression (L. Cao, R. Srikumar and K. Poole, unpublished data). Intriguingly, MexR levels are greatly increased in *nalC* strains (L. Cao, R. Srikumar and K. Poole, unpublished data), suggesting that MexR repressor activity is modulated in such mutants, perhaps in response to the increase in PA3720-3719 expression. Growth phase regulation of MexAB-OprM has also been reported in *P. aeruginosa*^{45, 201}, with expression increasing throughout growth and reaching a maximum in late log phase⁴⁵. This occurs independent of MexR⁴⁵, implicating yet additional regulator(s) in the control of *mexAB-oprM* expression.

2.1.3.2. MexCD-OprJ. Although inducible by a number of non-antibiotic substrates, MexCD-OprJ typically contributes to multidrug efflux in *P. aeruginosa* following overexpression of the efflux system as a result of mutations in the *nfxB* gene^{160, 161}. Occurring immediately upstream of the *mexCD-oprJ* genes, *nfxB* encodes a LacI-GalR family¹⁵¹ repressor of *mexCD-oprJ*¹⁷⁷ and is negatively autoregulated²⁰⁸. MexCD-OprJ hyperexpression in *nfxB* mutants, thus, results from depression of the efflux genes. Two classes of *nfxB* mutants have been described, expressing moderate (type A) or high (type B) levels of the efflux system, with resistance levels correlating with efflux gene expression¹³¹. The observation that the original *nfxB* mutant was not resistant to tetracycline or chloramphenicol⁷⁴, which is only seen in type B *nfxB* strains¹³¹, suggests that these two agents are poor substrates for this efflux system. At present it is unclear whether NfxB mediates the aforementioned drug induction of *mexCD-oprJ* expression, with, for example, drug interaction with NfxB alleviating repression of the efflux genes. Still, studies of drug induction of repressor-controlled multidrug efflux systems in other organisms have confirmed drug-repressor interactions leading to loss of repressor binding to efflux gene promoters and subsequent efflux gene expression^{64, 204}.

2.1.3.3. MexEF-OprN. The nature of mutation(s) leading to MexEF-OprN hyperexpression in *nfxC* strains have yet to be fully elucidated although it is dependent upon a functional product of the *mexT* gene located upstream of *mexEF-oprN*^{100, 128}. In some cases, *mexEF-oprN* hyperexpression results from reversion or intragenic suppression of *mexT* mutations present in some so-called wild type strains^{100, 128}. Still, in strains carrying wild type *mexT*,

mutation(s) in other, as yet unidentified genes appear to be important for *mexEF-oprN* hyperexpression¹²⁸. MexT is a LysR family²⁰² positive regulator of *mexEF-oprN* expression that is also responsible for the decrease in OprD expression seen in *nfxC* strains^{95, 156}. Preliminary studies indicate that MexT control of OprD occurs at the level of transcription^{95, 156}, although posttranscriptional control of OprD by MexT has also been suggested⁹⁵.

2.1.3.4. MexXY-OprM. A gene, *mexZ* (also called *amrR*²²⁵), has been identified upstream of *mexXY* (*amrAB*) and encodes a TetR family repressor of *mexXY* (*amrAB*) expression^{3, 225}. While mutations in *mexZ* (*amrR*) do provide for hyperexpression of the adjacent *mexXY* (*amrAB*) genes, this appears to be insufficient for aminoglycoside/MDR²²⁵, perhaps because additional necessary components are lacking or limiting. Thus, aminoglycoside resistance dependent upon MexXY/AmrAB hyperexpression in impermeability type mutants relies on mutations in genes in addition to or besides *mexZ/amrR*. Indeed, in a recent study of aminoglycoside resistance in clinical strains where resistance owing to MexXY overproduction was clearly evident, in no case was an inactivating mutation found in the *mexZ* (*amrR*) gene²¹⁰. While the most significant observation regarding regulation of *mexXY* is its inducibility by substrate antimicrobials, it is unclear whether this is mediated via the MexZ repressor (e.g., drugs target MexZ directly, obviating repressor activity thereby permitting *mexXY* expression, as has been seen for other drug-inducible efflux systems, see above). The additional, possibly regulatory gene(s) that are involved in MexXY-mediated acquired (i.e., impermeability type) aminoglycoside resistance may, in fact, mediate this antimicrobial induction of MexXY. Alternatively, *mexXY* expression may not be responding to the presence of antimicrobials directly, rather these indirectly promote efflux gene expression as a result of their impact on cell physiology (i.e., accumulation of specific cellular products in response to antimicrobial exposure up regulates MexXY and it is these products that are the intended substrates). Clearly, more work needs to be done both to define the complement of genes necessary for MexXY-mediated antimicrobial/aminoglycoside-resistance and to elucidate the nature of the drug inducibility of this efflux system.

2.1.3.5. Others. A gene encoding a TetR family repressor, *mexL*, has been identified upstream of the *mexJK* efflux genes (Figure 4) and shown to be a target for mutations that lead to hyperexpression of these genes³⁰. As expected, then, MexL has been confirmed as a repressor of *mexJK* expression³⁰. While a gene encoding a putative LysR family regulator has been identified upstream of the *mexGHI-opmD* genes (PA4203, Figure 4), there is as yet no evidence for a role in controlling efflux gene expression.

2.1.4. Efflux Inhibitors. Given the significance of RND family multidrug efflux systems vis-à-vis intrinsic and acquired resistance to antimicrobials, especially fluoroquinolones (reviewed in refs [170, 172]), in *P. aeruginosa* there is substantial interest in targeting these systems therapeutically as a way to enhance the antimicrobial susceptibility of this organism¹²¹. Indeed, efflux inhibitors effective against the Mex systems of *P. aeruginosa* have been described that are effective at overcoming efflux-mediated intrinsic and acquired resistance in this organism^{105, 120, 121, 150a, 150b, 150c, 189–191}. Significantly, too, these inhibitors appear to prevent the emergence of, for example, fluoroquinolone resistant strains *in vitro*, highlighting both the significance of these efflux systems for fluoroquinolone resistance and the potential effectiveness of efflux inhibitors in limiting resistance development in the first place¹²⁰.

2.2. Non-RND Systems

Very few multidrug efflux systems have been identified in *P. aeruginosa* that are not of the RND type, although the genome sequence does reveal many homologues of ABC, MATE, MF and SMR family transporters¹⁶³. A homologue of the MF family EmrB multidrug exporter from *E. coli* implicated in efflux of and resistance to a variety of energy uncouplers and the antimicrobial nalidixic acid¹¹⁸ has been identified in *P. aeruginosa* (see PA5159, Table 1; 48% identical to EmrB). EmrB functions as part of a tripartite efflux system that includes an MFP (EmrA) and OMF component (TolC)¹¹⁸ and PA5159 also occurs as part of a probable operon that includes both an MFP (PA5160, 44 percent identical to EmrA) and OMF (PA5158; a.k.a. OpmG) gene (Table 1). Whether this system is expressed or contributes to resistance in *P. aeruginosa* remains to be seen. Similarly, two homologues of the *Vibrio parahaemolyticus* NorM transporter of the MATE family, which exports a number of clinically relevant antimicrobials and appears to be well-conserved in Gram-negative bacteria¹⁷⁵, are present in the *P. aeruginosa* genome (NorM1 [a.k.a. PA1361] and NorM2 [a.k.a. PA5294]; Table 1). NorM1 was recently shown to encode functional efflux system, dubbed PmpM, that provided resistance to a number of dyes and antibiotics^{70a}. Recently, one of five candidate SMR type multidrug efflux systems in *P. aeruginosa* (PA4990, Table 1) has been shown to play a role in drug export¹⁰⁷. A homologue of the *E. coli* EmrE multidrug transporter of organic cations that include dyes and at least one biocide²⁰³ and thus dubbed EmrE_{PA}^{107, 153}, makes a modest contribution to intrinsic resistance to some dyes and aminoglycosides in *P. aeruginosa*¹⁰⁷. Finally, two genes encoding products with modest similarity (27% identity) to the *E. coli* MF family transporter MdfA that also exports a number of clinically relevant antimicrobials²⁰ are also present in the genome sequence (PA4136 and PA3573, Table 1). There is, however, no evidence as yet for an export function for these, let alone a role in drug resistance.

3. *PSEUDOMONAS PUTIDA*

P. putida is not generally a human pathogen, although the organism has gained a degree of notoriety as a result of the ability of certain strains to tolerate high concentrations of toluene^{185, 187}. In many instances this results from solvent efflux⁷⁹ by homologues of the RND-MFP-OMF multidrug efflux systems (see Table 1). The first to be reported, the solvent-inducible⁹³ SrpABC efflux system of *P. putida* S12, accommodates a variety of organic solvents although no medically relevant antimicrobial agents^{92, 94}. Two putative regulatory genes, *srpRS* (Figure 4) occur upstream of the efflux genes and disruption of *srpS* has been shown to enhance *srpABC* expression and solvent tolerance²²⁴, consistent with SrpS functioning as a negative regulator of efflux gene expression. Several non-motile transposon insertion mutants with disruptions in genes related to flagellum biosynthesis were solvent-sensitive and showed reduced expression of the *srpABC* genes⁹⁰ though the regulatory connection between flagellar biosynthesis and efflux gene expression is unknown. Similar mutants in *P. putida* DOT-T1E were also solvent-sensitive and impaired for solvent efflux²⁰⁷.

In contrast to SrpABC, the constitutively expressed MFP-RND-OMF type MepABC multidrug efflux system of *P. putida* KT2442 accommodates both organic solvents and antimicrobials⁵¹, reminiscent of the multidrug efflux systems of *P. aeruginosa* (see above). Indeed, the pattern of antimicrobial resistance afforded by MepABC is very similar to MexAB-OprM, the *P. aeruginosa* efflux system displaying the highest degree of amino acid sequence homology to the Mep proteins (66–70% identity)⁵¹. Virtually identical (to MepABC) efflux systems have been reported in *P. putida* strains DOT-T1E (TtgABC)¹⁸⁶ and S12 (ArpABC)⁹¹, and while TtgABC also affords intrinsic resistance to a variety of organic solvents^{186, 193} and antimicrobials^{38, 186}, ArpABC only promotes resistance to antimicrobials⁹¹. Still, this efflux system may, in fact, accommodate organic solvents but a contribution to solvent tolerance may be masked by the operation of other solvent efflux systems in strain S12. Expression of the *ttgABC* genes is negatively regulated by the TetR family product of the upstream *ttgR* gene³⁸ (Figure 4) identical versions of which have been identified upstream of *mepABC* (dubbed *mepR*) and *arpABC* (dubbed *aprR*) (Table 1). A recent study demonstrated that expression of *ttgABC* is induced by some of its antimicrobial substrates, apparently mediated by TtgR, which was shown to bind some of these^{218a}. Contrary to the Duque *et al.* report³⁸, however, *ttgR* is not under the control of an Lrp family global regulator dubbed TtgX¹⁸⁵ (Table 1).

Two additional solvent efflux systems of the RND-MFP-OMF type have been described in *P. putida* DOT-T1E and dubbed TtgDEF¹⁴³ and TtgGHI¹⁹³. The former is encoded by the *ttgDEF* genes linked to the *tod* genes of toluene metabolism and facilitates tolerance to a limited range of organic solvents but not resistance to antimicrobials^{143, 193} while the latter appears to be identical to

the SrpABC system of *P. putida* S12 (see above). The *ttgDEF* efflux genes are negatively regulated by the IclR family²⁴¹ product of an upstream and divergently transcribed gene, *ttgT*¹⁸⁵ (Figure 4), and as with the *srpABC* genes⁹³, the *ttgDEF* genes are inducible by organic solvents¹⁴³. An efflux system almost identical to TtgDEF, SepABC, has recently been described in *P. putida* strain F1 and appears to be present in a variety of *Pseudomonas* spp.¹⁶⁷. Under the control of a SepR repressor indistinguishable in sequence from TtgT, the *sepABC* genes are also linked to *tod* genes in strain F1, are solvent-inducible and do not provide resistance to antimicrobials¹⁶⁷. Still, the *speABC* genes are inducible by a range of organic compounds¹⁶⁷, suggesting that this system can accommodate many solvents, while the TtgDEF system seems to promote resistance to only a very limited range of solvents in DOT-T1E¹⁹³.

Genes virtually identical to *srpSR*, dubbed *ttgVW*, have been identified upstream of *ttgGHI* and as with knockouts in *srpS*, inactivation of *ttgV* also leads to increased efflux gene (i.e., *ttgGHI*) expression¹⁹⁴. The TtgV protein has, in fact, been shown to bind to overlapping promoters for *ttgVW* and *ttgGHI*, consistent with a role for this IclR family regulator in the negative control of *ttgGHI* (and *ttgVW*) expression¹⁹⁴. Like SrpABC, the TtgGHI efflux system promotes both intrinsic and solvent-inducible tolerance to toluene in wild type cells¹⁹³, though the former also provides intrinsic resistance to several additional solvents⁹² (i.e., loss of SrpABC in strain S12 compromises resistance to many solvents⁹² while loss of TtgGHI in strain DOT-T1E only compromises resistance to toluene and, possibly, styrene¹⁹³). This discrepancy is likely explained by the presence of additional, constitutively expressed solvent efflux systems (e.g., TtgABC, see above) in the TtgGHI-containing DOT-T1E strain of *P. putida* that may be absent in the SrpABC-containing *P. putida* S12. Indeed, mutants of DOT-T1E singly defective in either TtgABC or TtgGHI exhibit sensitivity to only a limited number of solvents while a mutant lacking both is sensitive to several solvents, indicating that both systems accommodate a range of solvents and that TtgABC can to some extent compensate for loss of TtgGHI.

4. *STENOTROPHOMONAS MALTOPHILIA*

An increasingly important nosocomial pathogen, particularly in debilitated or immunosuppressed individuals, *S. maltophilia* is resistant to multiple antimicrobial agents^{36, 52, 69, 184}. MDR attributable to efflux has been reported in this organism^{8, 238, 240} and, indeed, homologues of the MexB and OprM components of the MexAB-OprM multidrug efflux system of *P. aeruginosa* have been identified in clinical MDR strains of *S. maltophilia*²³⁸. As with *P. aeruginosa*, fluoroquinolones readily select MDR strains in vitro^{104, 238} although other agents such as tetracycline and chloramphenicol also yield MDR

strains^{8, 238}. To date, two RND-MFP-OMF type efflux systems, encoded by the *smeDEF*⁹ and *smeABC*¹¹¹ operons, have been described in this organism, with the former contributing to intrinsic multidrug resistance²³⁹ as well as resistance in clinical strains¹⁰. Although hyperexpression of *SmeABC* was observed in an *in vitro*-selected multidrug-resistant strain of *S. maltophilia*, only loss of *SmeC* compromised this resistance, suggesting that this OMF functions as part of another, as yet unidentified multidrug efflux system in promoting the observed resistance of this strain¹¹¹. A putative two-gene operon, *smeRS*, encoding homologues of the phosphorylation-dependent two-component signal transduction systems occurs upstream of the *smeABC* genes, with *SmeR* positively regulating efflux gene expression and, indeed, required for *SmeABC* hyperexpression and, thus, *SmeC*-dependent MDR¹¹¹. Expression of the *smeDEF* genes is under the negative control of the TetR family repressor, *SmeT*, whose gene lies immediately upstream of the efflux genes (Figure 4) and is the site of mutation in at least one *SmeDEF*-overexpressing multidrug-resistant isolate¹⁹⁸. Despite reports that a broad spectrum multidrug efflux inhibitor effective against *P. aeruginosa* (MC-207, 110; see above) enhanced the naladixic acid susceptibility of some clinical isolates of *S. maltophilia*^{192, 199} it did not compromise resistance to other known efflux substrates and was apparently inactive against *SmeDEF*¹⁹⁹. The impact of MC-207, 110 on naladixic acid susceptibility may, thus, reflect activity against a hitherto unidentified efflux system(s).

5. BURKHOLDERIA SP.

Originally described as the causative agent of soft rot in onions, *B. cepacia* is increasingly important as an opportunistic human pathogen, particularly in patients with cystic fibrosis or chronic granulomatous disease^{125, 212}. As with many other non-fermenting Gram-negative bacilli, the organism is intrinsically resistant to multiple antimicrobial agents and many clinical strains exhibit multidrug resistance^{17, 87}. Although the outer membrane barrier likely plays a significant role in this¹⁴, multidrug efflux pumps have also been implicated²⁴⁰. *CeoAB-OpcM* (Genbank accession number U97042) is an RND-MFP-OMF type MDR efflux system^{24, 25} that was first identified as a determinant of chloramphenicol resistance in a clinical strain²³. Providing resistance to trimethoprim and fluoroquinolones in addition to chloramphenicol, it is unclear whether this efflux system is only expressed in mutant strains or is also expressed constitutively, where it would contribute to intrinsic antimicrobial resistance. A multidrug efflux system of the MF superfamily, *BcrA*, has also been described in *B. cepacia*, providing modest resistance to a limited number of antimicrobials and unlikely to be of significance

clinically²²⁶. A RND-MFP-OMF type efflux system, AmrAB-OprA, has been described in *Burkholderia pseudomallei*¹³⁹, the highly antibiotic resistant^{139, 209, 229} causative agent of melioidosis³³. Also a multidrug transporter, this system exports and provides resistance to aminoglycosides and macrolides. Finally, a homologue of the MATE family multidrug efflux system first described in *Vibrio parahaemolyticus* and *E. coli*¹⁴⁰ has been described in *Burkholderia vietnamiensis* although it appears to contribute to resistance to polymyxin only and not to other antimicrobials⁴⁶

6. CONCLUDING REMARKS

Confirmed and probable efflux genes are common in *Pseudomonas aeruginosa* and related species and, indeed, in bacteria in general^{163, 170, 171, 173–176}. Given their involvement, particularly in the case of the RND-MFP-OMF pumps, in antimicrobial efflux and the current crisis regarding antimicrobial resistance in infectious disease^{47, 106} there has been scant attention paid to possible other non-antimicrobial substrates that in some instances might be the preferred or intended substrates. While virtually all of the hitherto known RND type efflux systems in *P. aeruginosa*, for example, accommodate antimicrobials, including many of the same antimicrobials, only one is antibiotic inducible (MexXY) and all are individually regulated by distinct regulatory genes (Figure 4). This suggests that each system has a unique function that is unrelated to their common contribution to antimicrobial export. While the solvent-inducibility and in some instances solvent-exclusivity of the *P. putida* efflux systems is consistent with an intended role for some of these in protection against solvents, the fact that some pumps, as in *P. aeruginosa*, accommodate both antimicrobials and solvents does raise questions about their intended substrates/function. The suggestion has been made that metabolic intermediates and/or by-products of metabolism may be the intended substrates of multidrug transporters in bacteria¹⁶⁹. The observation that auxotrophic mutations in *E. coli* leads to enhanced production of the AcrAB-TolC multidrug efflux system and, thus, produce multidrug resistance, probably owing to accumulation of metabolic intermediates that may be the intended efflux substrates, certainly supports this⁷². Clearly, however, the elucidation of the intended function of these will require that we look beyond their contribution to antimicrobial resistance and identify, for example, environmental signals that up/down regulate these systems and/or identify additional genes/proteins that are co-expressed with them (e.g., using a genomics/proteomics approach) and whose functions, if known, may provide clues as to the intended function of these efflux systems. Whatever, their intended function, however,

multidrug efflux systems are significant determinants of resistance in *P. aeruginosa* and other Gram-negative bacteria and as such good therapeutic targets for countering intrinsic and acquired antimicrobial resistance.

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THE FLAGELLAR SYSTEM OF *PSEUDOMONAS AERUGINOSA*

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1. INTRODUCTION

Pseudomonas aeruginosa typically possesses a single unsheathed polar flagellum that plays a variety of roles in the virulence in addition to its central role in swimming motility. The polar location of the flagellum of this organism (Figure 1A) contrasts with the peritrichously located flagella of enteric bacteria and the polar and lateral flagella found in some *Vibrio* spp. This polar placement is similar to that seen in some other monoflagellates, for example, *V. cholerae*, *Helicobacter* spp., *Campylobacter* spp., and a number of other pseudomonads. Besides the differences in the number and the location of the flagella from the well-described multiflagellated enteric bacteria, *Escherichia coli* and *Salmonella typhimurium* var. typhimurium⁴⁴, the regulation of the assembly of this complex structure in *P. aeruginosa* differs considerably from that seen in these organisms. These differences are the main focus of this chapter. General details of flagellar assembly can be found elsewhere^{2, 44}.

Based on electron microscopic morphology and the structural proteins found in the genome, the *P. aeruginosa* flagellum appears to be almost identical to that of enteric bacteria⁴⁴. Structural features are not described in great detail except for those features that may be of interest in studying the functions of the flagellum and those differences in the mechanism of its assembly that are strikingly different from that of the enteric bacteria. Among these are the existence of glycosyl groups on the flagellin of *P. aeruginosa* and heterogeneity

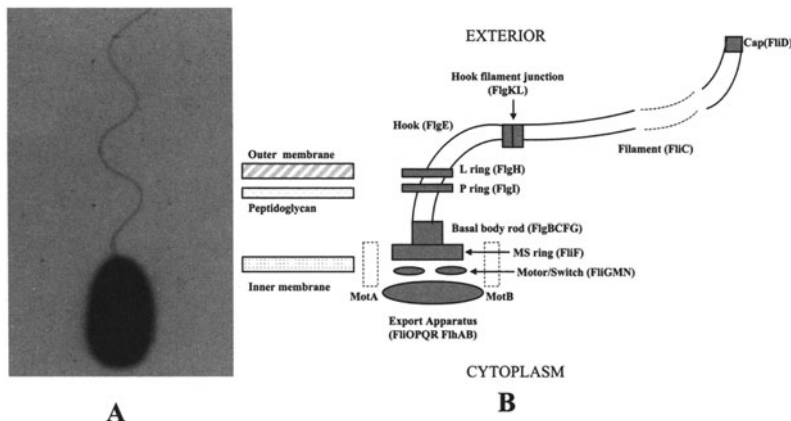


Figure 1. A. Monoflagellated *P. aeruginosa* cell with flagellum and B. Schematic structure of a flagellum showing main proteins and their cellular location.

of the genes encoding the major structural protein, flagellin. Additionally, the flagellar assembly pathway, which utilizes a different set of dedicated regulators at both the transcriptional and translational levels, some of which are shared by many of its monoflagellate cousins including other pseudomonads⁴⁵ and *Caulobacter crescentus*^{78, 79}, are also described. Although much new information on the flagellar system of *Pseudomonas aeruginosa* has been obtained in the last decade, the study of this organelle has lagged behind the study of the enteric systems and cannot be considered to be complete at this time. The information on the flagellar system of other pseudomonads is also incomplete but the emerging information from some genomes suggests that there will be some similar regulatory mechanisms and structural features in organisms such as *P. putida*, *P. fluorescens*, and *P. syringae*.

Besides the data on biogenesis that has been accumulated, an area of considerable interest is the role of the flagellum of bacteria in virulence. In *P. aeruginosa*, nonmotile strains were shown to be defective in virulence in certain models of infection⁵¹, but definitive evidence of a major role in virulence was only recently obtained from genetically characterized nonflagellate mutants²². Thus the possible functions of the proteins of the flagellum distinct from motility in virulence are also discussed.

2. STRUCTURE OF THE FLAGELLUM OF *P. AERUGINOSA*

The flagellum of *P. aeruginosa* is approximately 2.5 wave units in length. Each wave unit has a contour of 1.8 μm . However some cells may have a length of up to 5 wave units⁷⁰. As shown schematically, the flagellum consists

of three main components: A basal body embedded in the layers of the cell surface, a short curved hook, and a long external filament (Figure 1B). There is no flagellar sheath as seen with certain other organisms. Several other proteins are inserted along the flagellum to anchor it in the cell membranes and to provide a universal joint. Based on what is known from the *Salmonella* system, the structure is capped by a protein that prevents the secretion of flagellar proteins into the environment and aids in the polymerization of flagellin⁸⁰.

3. GENOMIC ORGANIZATION OF THE GENES BELONGING TO THE *fla* REGULON

In this section we have made an effort to consolidate the information available on the organization and regulation of the flagellar genes in various pseudomonads. However, most of the published work on the flagellar system in *Pseudomonas* is based on studies conducted in *P. aeruginosa*.

3.1. The *fla* Regulon

The completed *P. aeruginosa* strain PAO1 genome sequence⁶⁹, the commercially available *P. aeruginosa* genome array based on this sequence, and sequences from some other strains from the public genetic databases, has enabled the examination of the interplay of the various flagellar regulators in controlling flagellar gene expression. This has led to the construction of a putative flagellar assembly pathway that is described later. At the current time the annotated PAO1 genome contains 41 genes encoding structural or assembly components of the flagellum (Table 1). An additional nine genes known or predicted to be involved in chemotaxis are included in the table of annotations based on their linkage to flagellar genes in the regulon. The function of each gene product, elucidated from studies conducted in *P. aeruginosa* or predicted based on homology to proteins of known function found in other flagellar systems, is listed in Table 1. Of note there are several genes not found in enteric bacteria that are annotated in this table, *fleQ*, *fleSR*, *flhF*, *fleN*, *fleP*, and *fleL*. Additionally, certain genes that specify posttranslational modification of the *P. aeruginosa* flagellum are shown. These do not exist in the *fla* regulon of enteric bacteria, and are not found in all *P. aeruginosa* strains but similar genes may be present in some pseudomonads.

3.2. Nomenclature

Essentially, the names for genes having homologues in *Escherichia coli*/*Salmonella* spp. were adopted for *P. aeruginosa* in order to maintain the system of nomenclature and impart easy identification across genomes, for example, *fli*, *flg*, *flh*, *che*, etc. A *fle* prefix was chosen for genes that were not

Table 1. Annotation of *P. aeruginosa* flagellar biogenesis genes.

gene ^a	flagellin type ^b	reference sequence ^c		homology ^d (%)	function ^e
		type-b(PAO1)	type-a (PAK)		
Regulatory					
<i>flcQ</i>	a/b	PA1097	L81176	99	σ^{54} dependent transcriptional activator (E)
<i>flcN</i>	a/b	PA1454	AF 133657	100	flagellar number regulator (E)
<i>flcS</i>	a/b	PA1098	L41213	99	sensor kinase (H)
<i>flcR</i>	a/b	PA1099	L41213	99	σ^{54} dependent transcriptional activator
<i>flgM</i>	a/b	PA3351	AY029221	99	σ^{28} specific anti-sigma factor (E)
<i>fliA</i>	a/b	PA1455	X61231	99	σ^{28} sigma factor (E)
Structural					
<i>flgB</i>	a/b	PA1077	NA	NA	basal body rod (H)
<i>flgC</i>	a/b	PA1078	NA	NA	basal body rod (H)
<i>flgD</i>	a/b	PA1079	NA	NA	hook cap scaffold (H)
<i>flgE</i>	a/b	PA1080	NA	NA	hook (H)
<i>flgF</i>	a/b	PA1081	NA	NA	basal body rod (H)
<i>flgG</i>	a/b	PA1082	NA	NA	basal body rod (H)
<i>flgH</i>	a/b	PA1083	NA	NA	basal body L ring (H)
<i>flgI</i>	a/b	PA1084	NA	NA	basal body P ring (H)
<i>flgJ</i>	a/b	PA1085	unpublished	100	flagellum specific muramidase (H)
<i>flgK</i>	a/b	PA1086	AF332547	87	hook-filament junctional protein (H)
<i>flgL</i>	a/b	PA1087	AF332547	72	hook-filament junctional protein (H)
<i>fliC</i>	a/b	PA1092	M57501 (<i>flaA</i>)	70	flagellin (E)
<i>flcL</i>	a/b	PA1093	L81176 (<i>flaG</i>)	57	filament length control (E)
<i>fliD</i>	a/b	PA1094	L81176	58	filament cap, mucin adhesin (E)
<i>fliS</i>	a/b	PA1095	L81176	53	filament elongation (H)
<i>flcP</i>	a/b	PA1096	L81176 (<i>orf96</i>)	59	type IV pili length control (E)
<i>fliE</i>	a/b	PA1100	L43507	99	basal body component, MS ring/rod adapter (H)
<i>fliF</i>	a/b	PA1101	L43507	99	basal body MS ring, mounting plate for motor/switch (E)
<i>fliG</i>	a/b	PA1102	L43507 (partial)	99	motor/switch, mounted onto MS ring, rotor component (H)
<i>fliH</i>	a/b	PA1103	NA	NA	negative regulator of FliI (H)
<i>fliI</i>	a/b	PA1104	NA	NA	ATPase (H)
<i>fliJ</i>	a/b	PA1105	NA	NA	chaperone, export of hook proteins (H)
<i>fliK</i>	a/b	PA1441	NA	NA	hook length control (H)
<i>fliH</i>	a/b	PA1453	NA	NA	polar flagellar site determinant (H)
<i>flgA</i>	a/b	PA3350	AY029221 (partial)	99	P ring assembly component (H)
<i>flgN</i>	a/b	PA3352	AY029221	100	initiation of filament assembly (H)
Export apparatus					
<i>fliO</i>	a/b	PA1445	L39832	99	flagellar export pathway (H)
<i>fliP</i>	a/b	PA1446	L39832 (partial)	100	flagellar export pathway (H)
<i>fliQ</i>	a/b	PA1447	NA	NA	flagellar export pathway (H)
<i>fliR</i>	a/b	PA1448	NA	NA	flagellar export pathway (H)
<i>fliB</i>	a/b	PA1449	NA	NA	flagellar export pathway (H)
<i>fliA</i>	a/b	PA1452	NA	NA	flagellar export pathway (E)

Table 1. Continued

gene ^a	flagellin type ^b	reference sequence ^c		homology ^d (%)	function ^e
		type-b(PAO1)	type-a (PAK)		
Motor/switch component					
<i>fliM</i>	a/b	PA1443	L39832 (partial)	100	motor/switch (H)
<i>fliN</i>	a/b	PA1444	L39832	99	motor/switch (H)
<i>motA</i>	a/b	PA1460	NA	NA	motor rotation (H)
<i>mots</i>	a/b	PA1461	NA	NA	motor rotation (H)
Chemotaxis					
<i>cheY</i>	a/b	PA1456	X61231	100	switch regulator (H)
<i>cheZ</i>	a/b	PA1457	NA	NA	CheY phosphatase (H)
<i>cheA</i>	a/b	PA1458	NA	NA	CheY and CheB kinase (H)
<i>cheB</i>	a/b	PA1459	NA	NA	chemoreceptor methylesterase (H)
<i>cheW</i>	a/b	PA1464	NA	NA	positive regulator of CheA (H)
<i>cheV</i>	a/b	PA3349	NA	NA	chemotaxis regulator (H)
<i>cheR</i>	a/b	PA3348	NA	NA	chemoreceptor methylesterase (H)
Flagellin glycosylation					
<i>vioA</i>	a	NP	AF332547	NA	flagellin glycosylation (E)
<i>orfA</i>	a	NP	AF332547	NA	flagellin glycosylation (E)
<i>orfB</i>	a	NP	AF332547	NA	flagellin glycosylation (E)
<i>orfC</i>	a	NP	AF332547	NA	flagellin glycosylation (E)
<i>orfD</i>	a	NP	AF332547	NA	flagellin glycosylation (E)
<i>orfE</i>	a	NP	AF332547	NA	flagellin glycosylation (E)
<i>orfF</i>	a	NP	AF332547	NA	flagellin glycosylation (E)
<i>orfG</i>	a	NP	AF332547	NA	flagellin glycosylation (E)
<i>orfH</i>	a	NP	AF332547	NA	flagellin glycosylation (E)
<i>orfI</i>	a	NP	AF332547	NA	flagellin glycosylation (E)
<i>orfJ</i>	a	NP	AF332547	NA	flagellin glycosylation (E)
<i>orfK</i>	a	NP	AF332547	NA	flagellin glycosylation (E)
<i>orfL</i>	a	NP	AF332547	NA	flagellin glycosylation (E)
<i>orfM</i>	a	NP	AF332547	NA	flagellin glycosylation (E)
<i>orfN</i>	a	NP	AF332547	NA	flagellin glycosylation (E)
Unknown					
<i>fliL</i>	a/b	PA1442	NA	NA	unknown
<i>fliS'</i>	a	NP	L81176 (<i>orf126</i>)	NA	unknown

^aUnified nomenclature for genes encoding *P. aeruginosa* type-a and -b flagellar components and linked chemotaxis proteins. Genes are grouped according to their predicted function.

^bFlagellin type specifies whether an allele of the gene is present in the type-a (a) and/or type-b (b) reference strain.

^cThe source of the sequence for each gene is provided. Genes not present in a given reference strain are denoted NP. PA numbers refer to the unique identification number assigned to each gene as part of the PAO1 genome sequence annotation project (www.pseudomonas.com). GenBank accession numbers are given for strain specific sequences found in PAK. Parentheses indicate an alternative gene name was provided in the accession. NA indicates that the DNA sequence is not available for the PAK allele but that its existence is predicted based on the cross-hybridization of PAK genomic DNA to oligonucleotide probes derived from the corresponding PAO1 gene sequence.

^dGiven is the percent identity between type-a and -b strain alleles. NA signifies that only one sequence is available.

^eFunction of the gene product was assigned based on studies conducted in *P. aeruginosa* (E) or predicted based on homology (H) to proteins of known function found in other flagellar systems.

present in the enteric system and were first identified in *P. aeruginosa*, in order to facilitate their distinction, for example, *fleQ*, *fleN*, etc. This new prefix was initially utilized when the flagellar sensor-regulator pair *fleSR* were identified and found to be absent from the enteric system⁶². Homologues of some of these *fle* genes are now known to be present in *Caulobacter*, *Vibrio*, *Campylobacter*, *Helicobacter* where they have been given a different prefix and also in some other pseudomonads.

3.3. Chromosomal Organization of Flagellar Genes

Seventeen putative operons comprising 50 flagellar or linked chemotaxis genes in the *P. aeruginosa* PAO1 genome are clustered in three regions of the chromosome (Figure 2). The operon arrangements and other regulatory features are depicted. The rationale for assigning operon structures to groups of flagellar genes was based on the following criteria: (a) Previous demonstration of their existence in a transcriptional unit. (b) The lack of an appreciable intergenic region between genes. (c) The presence of a *rho*-independent terminator sequence. (d) Experimentally demonstrated activity of the putative promoter in the *P. aeruginosa* PAK background¹⁹.

Most of the structural genes coding for the basal body rod, rings, hook, filament (flagellin), cap, and basal body (*flgBCDE*, *flgFGHIJKL*, *fliCfleL*, *fliDS*, and *fliEFGHIJ*) are clustered in Region I, starting at 1,164,275 of the PAO1 genome. The transcriptional regulator *fleQ* and two-component system *fleSR* are also located within this region. Region II, starting at base pair 1,570,496 on the PAO1 chromosome, contains genes encoding the hook length regulator, switch, export apparatus, flagellar placement determinant, flagellar number regulator, and alternative sigma factor FliA (σ^{28}) (*fliK*, *fliLMNOPQRflhB*, *flhA*, *flhFfleN*, *fliA*). This region also includes genes encoding motor and chemotaxis proteins (*cheYZ*, *cheAB*, *motAB*, *cheW*). Region III (beginning at 3,761,960 bp in PAO1) consists of genes coding for the flagellar export apparatus, anti-sigma factor (*flgA*, *flgMN*) and additional chemotaxis regulatory proteins (*cheVR*). Whether there are other regulators that are solely dedicated to controlling flagellar biogenesis in other parts of the chromosome has not been excluded. Other genes that regulate flagellar assembly but have other functions, for example, *rpoN*⁷⁴ are not included as part of the regulon. It is likely that other such genes will be discovered.

The existence of a glycosylation island between the flagellar genes of Region I of *P. aeruginosa* is shown but as is discussed later, the number of genes present varies from strain to strain of *P. aeruginosa*. The chromosomal organization of the flagellar genes of *P. putida* and *P. syringae*, taken from the available genome data is also shown using the nomenclature used in the annotation of the *P. aeruginosa* genome (www.Pseudomonas.com). The organization of the regulons in the bacteria show some differences from that

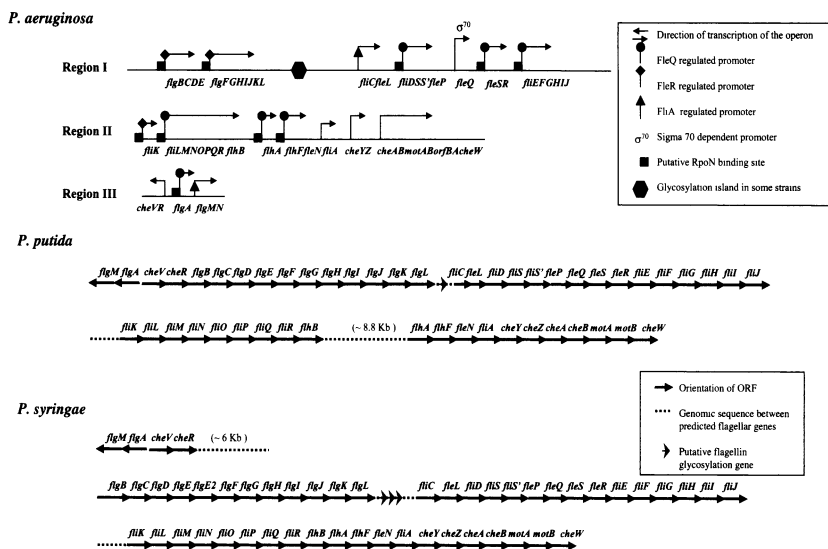


Figure 2. Chromosomal organization of the *fla* regulon in *P. aeruginosa*, *P. putida*, and *P. syringae*. In *P. aeruginosa* the genes of the *fla* regulon have been depicted in an operon format. The operon structure for *P. putida* and *P. syringae* are yet to be elucidated. Site of action of important regulatory proteins are shown as per legends in boxes. The region between *flgL* and *fliC* in *P. syringae* is most likely the location of glycosylation determinants in this species.

of *P. aeruginosa* as may be expected, but there do not appear to be any novel genes present in these. The organization is discussed below.

4. POLYMORPHISMS OF THE STRUCTURAL FLAGELLAR GENES OF *P. AERUGINOSA*

4.1. Differences between Strain PAO1 and Strain PAK

Most information on the flagella of other bacteria is based on single or a few sequences of any given gene. However, interest in the structure of the flagellins of *P. aeruginosa*^{8, 10, 67, 76} has allowed the accumulation of a large number of sequences that allows us to examine some polymorphisms. Most prominent among these is *fliC*, the gene encoding flagellin, the major component of the flagellum. The flagellar gene polymorphisms were believed to be limited to flagellin, which consisted of two types called type-a and type-b^{11, 67, 76}. However, much new information has been gathered on other *P. aeruginosa* flagellar genes, which indicates that the differences in these two types of flagella are more far-reaching. Another widely used laboratory strain PAK has been extensively studied and many of its flagellar genes were either completely or partially sequenced. The sequences of *flgGHIJKL*, *fliC*, *fliE*, *fliDSS'*, *fliP*, *fliQ*, *fliSR*, *fliEFG*, *fliK*, *fliL*, *fliM*, *fliN*, *fliO*, *fliP*, *fliN*, and

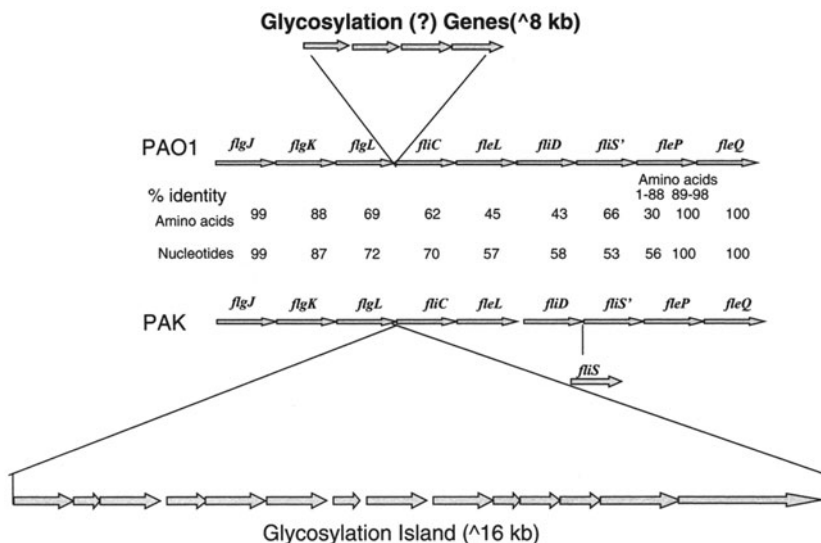


Figure 3. Polymorphisms of fla regulon of *P. aeruginosa*.

flgAMN, from strain PAK^{2-6, 16, 23, 62} (Accession numbers, AY029221, AF332547, AF139824, AF133657, L81176, L43507, L41213, and L39832) are now known. When these sequenced areas of PAK are compared to the homologous regions in the PAO1 genome, several differences are observed. As shown in Figure 3, this sequenced region of the flagellar regulon is highly polymorphic, at least between *P. aeruginosa* strains PAO1 and PAK². On the 5' end, *flgJ* sequences of PAK and PAO1 were nearly identical and homology of the genes following *flgJ* dropped gradually and then dramatically. On the 3' end, a precise end of this polymorphic sequence could be identified in the *flhP* gene where the first 264 nucleotides of *flhP* were only 56% identical between PAK and PAO1 but the last 31 nucleotides of *flhP* and the complete sequences of the regulatory genes, *flhQ* and *flhSR*, were 100% identical. The comparison of the sequences in this region suggests that horizontal gene transfer to PAK and PAO1 strains may be responsible for this sequence variability and that the most likely sites of recombination exist somewhere in the coding sequences of *flgJ* and *flhP* genes.

4.2. Polymorphisms among the Glycosylation Island of Different *P. aeruginosa* Strains

Further analysis of this polymorphic region 1 in strain PAK indicated the presence of an island consisting of 14 open reading frames (ORFs) inserted in

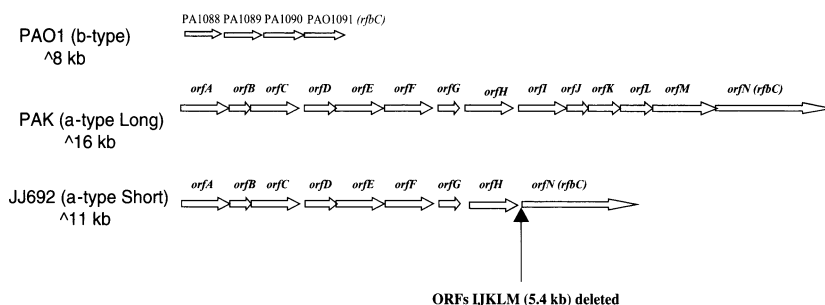


Figure 4. Schematic diagram showing the structure of GIs of a- and b-type *P. aeruginosa* strains. The diagram shows the region of the *P. aeruginosa* chromosome where the GIs are located: The 3 types of GIs are shown b-type PAO1 with 4 ORFs, a-type with complete GI (Long), a-type with a deletion of ORFs IJKLM (Short). The location of the GI insertion is shown to be in the middle of the flagellar genes *flgL* on the 5' end and *fliC* at the 3' end.

the middle of a cluster of flagellar genes. Some of the genes of this island, possible the whole island is involved in glycosylation of PAK flagellin² By contrast, in strain PAO1, three ORFs of unknown function and one ORF having significant homology to an *rfbC* gene (coding for a homologue of an O antigen biosynthesis protein) are present in this region⁶⁹. Another abbreviated version of this glycosylation island, having a large deletion in the middle is now also known to be present in *P. aeruginosa* strains from diverse origins carrying the type-a flagellin⁹ giving rise to a shorter island (Figure 4). The extent of the variability of the oligosaccharide modifications on flagellin is not currently known (discussed in detail below), however since the type-a flagellins show such variability in their apparent masses¹¹, it can be expected that further polymorphisms that specify different oligosaccharide structures may exist in this region.

4.3. Flagellin and Cap Protein Modifications

The gene encoding the type-b flagellin is highly conserved leading to expression of a protein that has an almost invariant amino acid sequence from strain to strain¹¹. However sequencing of a number of type-a flagellin genes indicated that the central region of these genes are heterogeneous^{11, 67}. One classification of the type-a genes, based on restriction digests suggests that there are at least 13 subtypes of the type-a flagellin⁷⁶. Another attempt at classification indicated that among 12 type-a strains there were six groups based on diagnostic nucleotide patterns at dimorphic sites⁶⁹. However a closer examination of the amino acid sequence of the type-a flagellins suggests that there are only two major subtypes of flagellin protein that differ from each other by the presence of two small deletions in the central region of the protein (Figure 5)⁹. The other minor structural differences involve amino acid substitutions in each type of

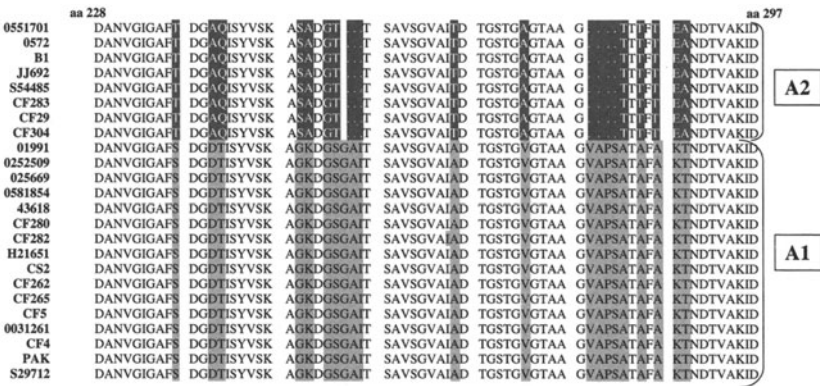


Figure 5. PILEUP analysis of the partial central domain of 24 *P. aeruginosa* strains. Amino acid (aa) sequences from aa228 to aa297 of a-type flagellins of 24 *P. aeruginosa* strains were aligned using the PILEUP program of the GCG Wisconsin package. Amino acid substitutions at positions 237, 240, 241, 249, 250, 253, 266, 273, 283, 285, 287, 288, and 289 and two small deletions of 3 and 4 amino acids respectively at positions 254 and 279 are highlighted in different shades of gray.

protein. These subtypes have tentatively been called flagellin subtypes A1 and A2. Similar sequence polymorphism are now known to exist in the flagellins in other Gram-negative organisms such as *H. pylori* and *E. coli*^{32, 75}.

Another feature of the polymorphism of the flagellar genes of *P. aeruginosa*, is the possession of two types of flagellar cap proteins, Flid, denoted as type A or B, which were only 58% identical at the nucleotide level and 43% identical at the amino acid level³. These genes are co-inherited with their cognate flagellin gene types, a or b, suggesting lateral gene transfer from other organisms rather than evolution from one form to another. One interesting aspect of this co-inheritance is the inability of one type of cap to function as efficiently as when paired with a heterologous flagellin, implying a strict stereo specific association between these proteins³. These proteins, which also function as adhesins for respiratory mucins, are also likely to recognize different oligosaccharide structures⁶⁴ based on this polymorphism.

5. GLYCOSYLATION AND PHOSPHORYLATION OF *P. AERUGINOSA* FLAGELLIN

Glycosylation of bacterial proteins is a relatively new finding since glycosylation is generally considered to be a more common characteristic of eukaryotic organisms. However, in the past few years several reports on prokaryotic glycosylation have appeared in the literature^{10, 11, 49, 60}. More specifically, glycosylation of not only archaeobacterial flagellins but also many pathogenic and non-pathogenic eubacterial flagellins has been demonstrated^{27, 33, 72}.

Glycosylation of *P. aeruginosa* type-a flagellins was first demonstrated by Brimer and Montie in 1998¹¹ and was followed by identification of a glycosylation island consisting of 14 ORFs, which had homology to many fatty acid biosynthesis and lipopolysaccharide biosynthesis genes². At least two genes present at either end of this cluster were shown to be essential for the glycosylation of type-a *P. aeruginosa* flagellin. However, the nature of the glycan added to the flagellin subunits was not known and the function of the individual genes in the island was unknown. Recently, mass spectrometry was employed to identify the sites of O-linked glycosylation in the PAK flagellin⁴³. Two amino acids in the predicted protein, serine 190 and threonine 261 have been found to be "O" glycosylated. The oligosaccharide chain extends from 6 to 11 residues. The glycan is attached to the peptide backbone via a deoxyhexose residue and capped by a unique trisaccharide. This complex carbohydrate seems to be unique to *P. aeruginosa* since the flagellins of two other pathogenic bacteria, *Campylobacter* and *Helicobacter*, were both found to carry pseudaminic acid as the added sugar^{67, 73}. At the current time it is certain that the sugars are not the same sugars that are found on the "O" side chain of serotype 6 (strain PAK). Type-b strains were thought to lack the glycosylation sites and the pathway for glycosylation, however, very recent studies using mass spectrometry suggests that the prototype strain PAO1 is also post-translationally modified with a mass of 700 Da⁷, which is greater than the expected phosphotyrosine modifications that has been described³⁸. The existence of a glycosyltransferase just before the flagellin gene suggests that glycosylation may be responsible (PAO 1091; Figure 3) for the extra mass. The functions of the glycosyl groups in the type-a strain PAK are not linked to flagellar assembly or motility since mutations in the glycosylation island that abrogate glycosylation did not affect either of these². Some early information suggests that the glycosyl groups may play a role in virulence in the burn mouse model of infection⁸. Tyrosine phosphorylation of both type-a and type-b flagellins has also been described. Their genetic basis is not known. There appears to be four phosphate groups on each molecule of type B flagellin and three on the type-a molecule³⁸. The function and genetic basis of this modification is currently unknown.

6. REGULATION OF FLAGELLAR ASSEMBLY IN *P. AERUGINOSA*

Flagellar assembly in bacteria is an exquisitely timed process designed to economize on cell resources and to avoid unnecessary and wasteful synthesis of the flagellar proteins. As such it is subject to tight transcriptional, post-transcriptional, and posttranslational regulation at DNA, RNA, and protein levels¹. The transcriptional process is generally hierarchical involving several classes of genes in most flagellated bacterial species studied, including *E. coli*, *S. typhimurium*⁴⁴, *C. crescentus*^{78, 79}, *V. cholerae*⁵⁹, and *V. parahaemolyticus*⁴⁶.

In the observed trend, transcription of the class higher up in the hierarchy is required before transcription of the subsequent class lower down in the hierarchy. The enteric paradigms, *E. coli* and *Salmonella*, utilizes three classes of genes namely, Class 1, 2, 3 (a, b subclass), in their transcriptional hierarchical layouts. Many of these genes are transcribed from multiple promoters. Thus, to avoid confusion the genes were alternatively grouped as early, middle, and late¹³. *C. crescentus* another extensively studied model of assembly utilizes four classes⁷⁹. *P. aeruginosa* is similar to *Caulobacter* in several respects but with some notable differences such as the possession of the *flgM* gene²⁴ and the presence of a second two-component regulatory system⁶². Among the transcriptional hierarchical schemes published from different bacterial species for the flagellar genes, the scheme for the *Vibrio* spp.^{46, 59} bears the closest resemblance to that of *P. aeruginosa*.

6.1. Transcriptional Hierarchy of the *P. aeruginosa* Flagellar Regulon by Gene Class

Transcriptional profiling was used to compare the expression of genes of the flagellar regulon in wildtype and isogenic *rpoN*, *fleQ*, *fleR* and *fliA* non-polar deletion. Strain PAK, a type-a *P. aeruginosa* strain¹⁹, utilizes a four-tiered hierarchy involving the RpoN, FleQ, FleR, FliA, and FlgM regulatory proteins to control and coordinate the transcription of the flagellar regulon. Therefore *P. aeruginosa* flagellar genes have been grouped into four classes (Figure 6) in contrast to the enteric system that possesses three classes.

6.1.1. Class I Genes. The earliest genes are called Class 1 and their transcription is the first dedicated step in flagellar gene transcription. Among the flagellar genes, the transcription of *fleQ*, an NtrC like transcriptional activator⁵ and *fliA*, the flagellin specific sigma factor⁶⁸, are not influenced by any of the gene mutation(s) of known regulators, therefore their promoters are grouped as Class I. It is possible that other regulators, outside of the flagellar regulon do exist and influence these genes since at least one gene has been discovered to influence *fleQ* negatively¹⁷. It has not been possible to define a cognate sensor for the transcriptional regulator FleQ, however, there is evidence indicating the involvement of σ^{70} in the transcription of the *fleQ* gene and its repression by Vfr, the *P. aeruginosa* homologue of *E. coli* CRP¹⁷. The binding sites for Vfr and σ^{70} have a partial overlap, the binding of one may exclude the other thereby regulating the transcription of *fleQ*.

Caulobacter crescentus and enteric bacteria possess CtrA⁶⁰ and FlhCD⁴² respectively, which are considered to be the master regulators of flagellar biogenesis in these organisms. No such homologues have been found in *P. aeruginosa*, therefore FleQ is considered to be the master regulator of the flagellar regulon since it directly or indirectly regulates the expression of the majority of

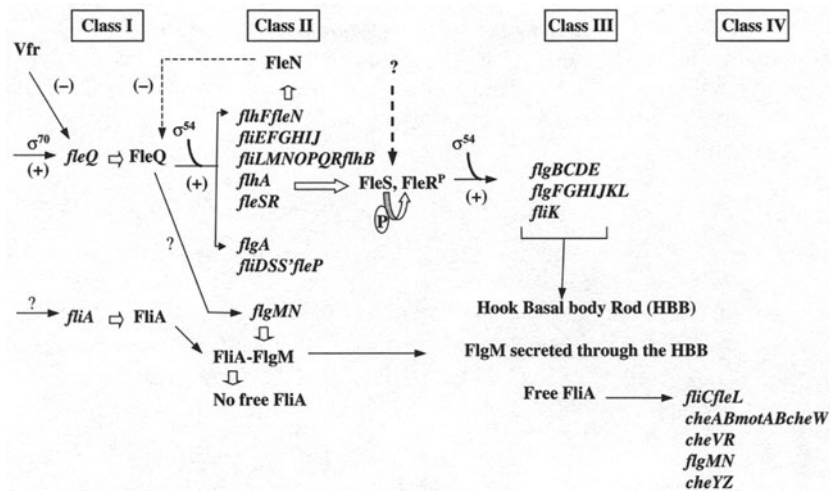


Figure 6. Transcriptional hierarchy of the various flagellar genes (Class I–IV) in *P. aeruginosa*. (–) and (+) denote negative and positive regulation respectively. ? denotes an unknown factor(s). Phosphorylation of FleR by FleS is denoted by transfer of phosphate (P).

flagellar gene promoters¹⁹ (Figure 6) with the exception of *fliA*. FleQ has been shown to directly bind to upstream regulatory elements and activate transcription of some structural genes including *flhA*, *fliE*, *fliL*³⁵. The molecular mechanism that controls the expression of *fliA* still remains unclear. The transcription of *fliA* appears to be constitutive and not dependent on σ^{54} or other flagellar regulators¹⁹ (Figure 6). Its transcription may therefore be initiated by a novel mechanism, perhaps involving another as yet uncharacterized sigma factor.

6.1.2. Class II Genes Require FleQ and σ^{54} . Promoter fusions and microarray studies indicate that *fliDSS'*, *fleP*, *fleSR*, *fliEFGHIJ*, *flhA*, *flhF*, *fleN*, *flgA*, and *fliLMNOPQR*,^{6,19} depend only on the product of the *fleQ* gene and σ^{54} and consequently are classified as Class II genes (Figure 6). The Class II genes encode structural components of the basal body, MS ring, P ring, motor, switch, flagellar export apparatus, and the filament cap. Regulatory proteins expressed from Class II genes include FleS and FleR, FlhF, and FleN (Table 1). Homologues of these regulatory proteins are missing in the peritrichous flagellar systems of *E. coli* and *S. typhimurium*. FlhF is the polar flagellar determinant in *P. putida*⁵⁶ and its close homologues are present in the genomes of a variety of mono-flagellated Gram-negative microorganisms. It is very likely that FlhF determines flagellar localization in other polar flagellates including *P. aeruginosa*. FleN, the anti-activator of FleQ, down-regulates FleQ activity through direct interactions and thereby plays a crucial role in maintaining a single flagellum^{16,18}. FleS and FleR comprise a two-component

system in which FleS is a sensor kinase for the response regulator FleR⁶². In *V. cholerae*, the FleS homolog FlrB activates the FleR homolog FlrC by phosphorylation¹⁵. Thus, the phosphorylation-dependent activation of FleR by its cognate sensor kinase FleS is likely to be necessary for the transcriptional progression from Class II to Class III promoters and may serve as an additional checkpoint in the flagellar biogenesis process in *P. aeruginosa*. This checkpoint is missing in the enteric paradigm. The majority of sensor kinases are integral membrane proteins, which activate their cognate response regulators in response to external stimuli. Analysis of the predicted secondary structure of FleS indicates that it is a cytoplasmic protein⁶². The signal sensed by *P. aeruginosa* FleS is unknown; given its probable localization to the cytoplasm, the signal is unlikely to originate from the extracellular environment perhaps it senses the completion of a structure or excess of a structural intermediate.

6.1.3. Class III Genes Require Activated FleR and σ^{54} . The promoters of operons, *flgBCDE*, *flgFGHIJKL*, and *fliK* are grouped under Class III as their expression required RpoN, FleQ, and FleR¹⁹ (Figure 6). The apparent involvement of FleQ is an indirect consequence of the *fleSR* promoter being FleQ dependent (Class II). The expression of the *fleSR* promoter also requires RpoN as do all other Class II promoters. In addition to its indirect effect, RpoN is also required for the expression of Class III promoters as evidenced by the identification of σ^{54} binding sites in each promoter element (Figure 2). Class III promoters control the expression of genes coding for the basal body-rod, L ring, hook, hook-cap scaffold, and hook-filament junctional proteins (Table 1).

6.1.4. Class IV Gene Expression. The absence of functional FliA (σ^{28}) results in a decrease in the expression from promoters of genes for the *fliCfleL* and *flgMN* operons. Both the promoters for the *fliCfleL* and *flgMN* operons possess a σ^{28} binding consensus sequence and are known to be transcriptionally dependent on FliA (σ^{28})^{24, 68}. Unlike the other Class IV gene, *fliC*, which lost most of its promoter activity in the *rpoN*, *fleQ*, *fleR*, and *fliA* mutants, the *flgMN* promoter retained partial transcriptional activity in all the mutant backgrounds¹⁹. A similar observation with the *flgMN* promoter was made in an earlier report²⁵. This indicated that the observed basal level of its transcription was independent of RpoN, FleQ, FleR, and FliA. It probably depends on another unknown transcriptional or sigma factor not yet discovered. The regulation of *flgM* is multifactorial in the enteric system. In *S. typhimurium*, the *flgM* promoter is dually regulated by FlhCD and FliA and is classified under Class 2 and 3a respectively⁴⁴. It is possible that the FliA independent synthesis of FlgM in *P. aeruginosa*, serves to regulate the transcriptional activity FliA and inhibits its activity until FliA is needed. Following the simultaneous expression of other Class II and III genes that contribute to the completion of the hook-basal body rod structure, FlgM is secreted by a similar mechanism as described for *S. typhimurium*³⁷ allowing for Class IV gene activation by free FliA (Figure 7).

6.2. Modulation of FliA Activity by the Anti-Sigma Factor FlgM

In organisms encoding FlgM and FliA, it has been established that FlgM directly binds to FliA and prevents it from activating transcription of FliA-dependent promoters^{1, 54}. The inhibition is postulated to be reversed following secretion of FlgM through the hook-basal body rod structure as demonstrated in *S. typhimurium*³⁷. Since the synthesis of components of the hook-basal body rod structure in *P. aeruginosa* is dependent on FleQ, FleR, and RpoN (Figure 7), the secretion of FlgM would be compromised in a FleQ or FleR mutant background. FlgM accumulation in such mutants would inhibit transcription from FliA dependent promoters, thus explaining the indirect regulation of *fliCfleL* and *flgMN* by the two transcriptional regulators and RpoN that was noted in transcriptional profiling.

6.3. Completion of the Hook Substructure Serves as a Checkpoint for the Transcription of FliA Dependent Genes such as *fliC*

In *P. aeruginosa* there also appears to be a similar mechanism involving the interaction of FlgM and FliA as in *S. typhimurium*, which couples the completion of the HBB structure to flagellin expression¹⁹. Based on the transcriptional hierarchy scheme of flagellar genes and the assembly process of the

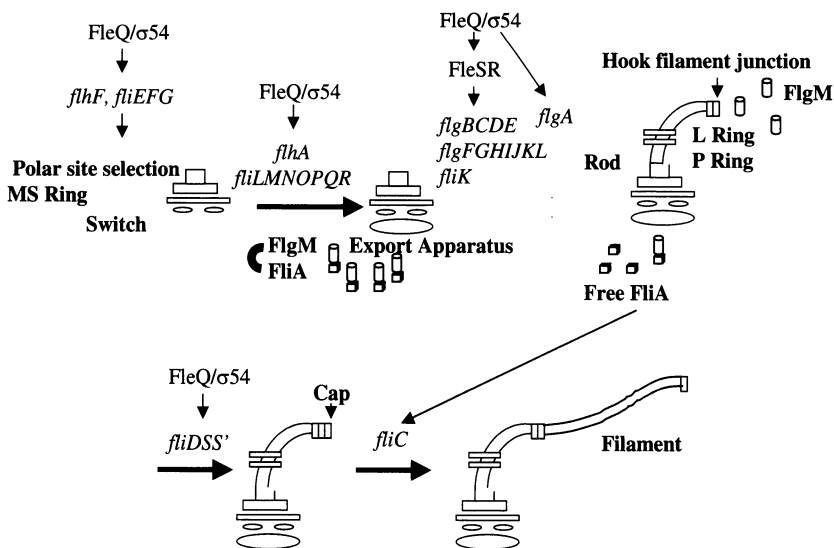


Figure 7. Model proposed for flagellar assembly in *P. aeruginosa*.

various flagellar components (Figures 6 and 7, and Table 1), completion of the HBB structure requires the expression of genes in Classes I–III. Western analysis conducted to semi-quantitatively detect flagellin in lysates of the *fleQ*, *fleR*, *fliA*, *flhF*, *fliF*, and *flgE* mutants revealed that FliC synthesis was compromised in them to variable degrees¹⁹, all of which predictably lack a functional HBB structure. Of these *fleQ* and *fliA* belonged to Class I, *flhF*, *fliF*, and *fleR* to Class II, and *flgE* to Class III, respectively. Presumably, in the absence of a functional HBB structure in these mutants, FlgM is unable to be secreted out into the medium and accumulates intracellularly. This accumulation results in the inhibition of FliA activity by direct association of FlgM with FliA preventing gainful expression of *fliC*.

7. MODEL PROPOSED FOR FLAGELLAR ASSEMBLY IN *P. AERUGINOSA*

In free-swimming *P. aeruginosa*, FleQ is presumed to be the master regulator of flagellar biogenesis. However, there is no distinctive consensus sequence in the FleQ binding sites of the FleQ regulated Class II genes examined³⁵. The lack of a FleQ binding consensus in these gene promoters suggests that FleQ probably has variable affinity for the different promoters it controls. This may represent a mechanism by which this single transcription factor could control the timing and level of expression of various components required at different stages in the flagellar assembly process. A similar mechanism controlling the expression of flagellar gene promoters has been described in *E. coli*³⁶. Thus while FleQ regulates expression of directly or indirectly of genes Classes II–III, their timed expression to allow assembly of the components may be in part controlled by the differences in promoter affinity.

The proposed model for the assembly of the single polar flagellum in *P. aeruginosa* presented in Figure 7 is based on the present understanding of transcriptional and posttranslational regulation of the various flagellar genes. FleQ transcription begins under the control some unknown stimulus, for example, cell cycle cues and results in the synthesis of Class II gene products. Initiation of assembly is most likely to begin with FlhF determining the polar placement site for the new flagellum. FlhF is thus an example of a Class II gene product that is required early on in the biogenesis of this organelle. The other Class II gene products comprising the MS ring, switch, basal-body, and export apparatus are subsequently assembled in the maturing flagellum. FliD, the filament cap protein, though expressed from a Class II gene is probably not assembled into the growing flagellar organelle until the hook and the hook-filament junctional proteins expressed from Class III genes are incorporated into the structure. Meanwhile, simultaneous synthesis of FleN, another Class II gene product, helps maintain the monoflagellate status by inhibiting

FleQ activity and down-regulating further synthesis of most of the structural components. The FleSR two-component system subsequently activates the Class III genes coding for proteins needed for the completion of the hook-basal body structure. This allows secretion of the anti-sigma factor FlgM and consequent transcription of FliA dependent genes coding for flagellin (FliC) and some chemotaxis proteins. The flagellar filament with the attached cap protein lengthens until *fleL*, a homologue of *Vibrio* spp. *flaG*, which is co-transcribed with *fliC* and which controls filament length⁴⁷, stops flagellar growth. Another protein the *Pseudomonas* specific Class II gene product, FleP then influences stability of the mature flagellar filament by an unknown mechanism. Where it acts and whether it is anywhere in the structure of the filament is not known. Other unknown steps are the location of the glycosylation apparatus and how this is regulated.

8. VIRULENCE FUNCTIONS OF THE *P. AERUGINOSA* FLAGELLUM

Bacterial flagella are commonly considered as organs of motility. Thus a virulence function for this structure has been ascribed to any role that motility may have in virulence. It was recognized several decades ago that non-motile strains of *P. aeruginosa* were less virulent than motile strains⁵⁰ but the basis of this attenuation was implied in relation to a lack of motility function. That specific virulence functions could be mediated by specific proteins of the flagellum, was not a mechanism of pathogenicity that garnered much attention. Since then other mechanisms of flagellar mediated virulence have been described for a number of bacterial flagella³⁴. Roles in virulence are now being described for several *P. aeruginosa* flagellar proteins that include the mediation of attachment²⁸, invasion²³, and mediation of an inflammatory response¹⁴. Additionally, the role of the *P. aeruginosa* flagellum in biofilm formation has now been well documented⁵⁵ adding another possible function this organelle may play in virulence.

8.1. Adhesion

Adhesion of *Pseudomonas aeruginosa* has been a subject of much investigation. Pilus-mediated adhesion has been reported in several studies^{61, 77} but there is increasing evidence that the flagellum is also involved. Flagellin mediated adhesion to corneal cells²⁸ has been reported, and flagellin has been implicated in adhesion to cell bound mucins⁴¹. The flagellar cap protein, FliD, has been shown to be a specific adhesin for respiratory mucins⁶, and to bind to specific oligosaccharides that are present in respiratory mucins⁶⁴. While it appears counterintuitive that an organelle that is involved in movement may

actually cause stickiness, there is much supportive evidence implicating bacterial flagella in adhesion in studies with other bacterial flagella^{26, 71}.

8.2. Invasion

FlhA of *P. aeruginosa*, a protein involved in the export of flagellar proteins, is a homologue of *Yersinia* spp. InvA that is believed to play a role in invasion of cells²⁵. Whether the homology is based strictly on both proteins being type III secretion protein homologues or a functionally similar was examined in the corneal model of *Pseudomonas* invasion. A *flhA* mutant is defective in corneal invasion, more so than a *fliC* mutant, which is also non-motile²³.

8.3. Inflammation

Innate immune responses to pathogen associated molecular patterns are now proving to be the basis of the inflammatory response to microbes³¹. The specific response to flagellin has become an exciting area of investigation for a variety of reasons. This response is not peculiar to *P. aeruginosa* flagellin but appears to be shared by all flagellins examined, including the response of plants to the flagellins of *P. syringae*, the so-called HRP response⁷². The innate immune response to flagellins in animals is mediated via toll-like-receptor 5 TLR5 and in the case of *P. aeruginosa* flagellin, the TLR5 binding site has now been isolated to a leucine-rich repeat region on this molecule⁴⁸. No specific studies with *P. aeruginosa* have identified the region of the flagellin molecule that is responsible for this interaction, but it is predicted to be a stretch of amino acids in the N-terminal region of the molecule³⁰. The clinical importance of this interaction is now being explored with initial observations that *Pseudomonas* flagellin is more potent than *Pseudomonas* LPS in causing inflammation when instilled into the lungs of mice⁴⁰.

9. IMMUNOLOGICAL RESPONSES TO FLAGELLIN

Immunological responses to *P. aeruginosa* were used as a means of classifying and typing strains during the 1960s and 1970s. Since that time this approach has given way to more modern approaches such as the use of DNA probes or RFLP typing of *Pseudomonas* strains^{51, 53}. However given the current interest in a flagellin-based vaccine against *P. aeruginosa*^{20, 21} an understanding of the immunological responses would seem to be important. Serotyping of *P. aeruginosa* on the basis of the immune response to flagella yielded two schemes. The initial scheme proposed by Lanyi described two serotypes-a and-b⁴². The other typing system classifies flagella into H serotypes⁶⁰ suggesting that there may be a least six serotypes. Cross-reactions

among serotypes appear to be extensive, with the exception of the H3 serotype that does not cross react with others. The H3 serotype is now known to be represented by strain PAO1, which is a type-b strain, strains that are known to be almost invariant in their primary amino acid sequence^{11, 67}. Other serotypes are likely variants of the type-a strains, since Serotype H4 represented by strain PAK a type-a strain shows extensive cross-reactions with other serotypes⁵⁷. Evidence of the immunological response to flagellin appears to be quite conclusive. Flagellar preparations used as an immunogen in mice confers protection in the burn mouse model²⁹. Monoclonal antibody against flagellin has been shown to be protective in an animal model⁵². These data suggested the feasibility of a flagellin-based human vaccine. Human studies of a flagellar vaccine are now in progress in patients with cystic fibrosis²⁰.

10. FLAGELLAR SYSTEMS OF OTHER PSEUDOMONADS

Among other *Pseudomonas* spp., flagellar genes have been studied in *P. putida*⁵⁶, *P. syringae*⁶⁶, and *P. fluorescens*^{12, 63}. However a systematic study has not been done in any of these species to ascertain whether there are significant differences from *P. aeruginosa*. Recently, the genome sequence of *P. putida* (GenBank Accession no. AE015451), a soil bacterium, and *P. syringae* (GenBank Accession no. AE016853), a phytopathogen, have been completed. Since there is no published report on the organization of the flagellar genes in these species, we have attempted to make a comparison of the basic organization of the *fla* regulon in these two organisms to that of *P. aeruginosa*. The organizational scheme in *P. putida* is schematically represented in Figure 2. In *P. putida* and *P. syringae*, all of the flagellar and linked chemotaxis genes are clustered in one region. The genes corresponding to Regions I and II of *P. aeruginosa* map in a continuous locus maintaining the same sequential order whereas those belonging to Region III map upstream of *flgB*. In *P. putida*, there is an 8.8-kb gap between *flhB* and *flhA*, whereas in *P. syringae*, a 6-kb gap is found between *flgM* and *flgB*.

The *flhF* gene of *P. putida* when disrupted leads to the loss of directional motility and random flagellar arrangement, implicating FlhF's role in polar flagellar placement. The same mutant in addition was also compromised in the development of the starvation-induced general stress resistance (SGRS) and displayed an altered protein synthesis pattern. Among pseudomonads, studies on chemotaxis have used *P. putida* as the preferred model and is discussed in a separate chapter. Little work has been published on the flagellar systems of other pseudomonads but in *P. fluorescens*, AdnA has been identified as the homolog for the FleQ transcription factor. Its disruption affects flagellar

synthesis, biofilm formation, and sand adhesion¹². This system is therefore likely to be similar to the *P. aeruginosa* paradigm.

11. CONCLUSIONS

The flagellar systems of pseudomonads are now of much interest in biology and medicine. The importance of these systems for free-living environmental bacteria that inhabit a variety of hostile niches cannot be overstressed. Besides being motility systems that allow for chemotaxis toward nutrients, these systems are used by these organisms to colonize and survive in a variety of locations, from the human lungs to plant and rock surfaces. The variable role of these organelles encompass include the ability to form biofilms in nature, to allow adhesion to a variety of substrates, and to mediate inflammation in both plants and animals. Glycosylation of flagellins, one newly described feature of these organelles occurs in at least two species of pseudomonads. There is already evidence to suggest that this may play a role in virulence and inflammation. A study of this particular phenomenon is likely to open new avenues to understanding microbial physiology since there is no “Golgi” apparatus in bacteria where this would occur. This chapter has summarized what is known mainly about the flagellar system *P. aeruginosa*, undoubtedly it is incomplete and other genes out of the *fla* regulon that may influence motility remain to be described as well as information about the flagellar systems of the other pseudomonads.

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MECHANISMS OF ADHESION BY PSEUDOMONADS

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1. INTRODUCTION

Pseudomonads can be infectious agents, biocontrol strains or commensal organisms and are found everywhere in the environment. How do they survive in these diverse environments? One way is by attaching to and colonizing a surface. Pseudomonads use adhesins to initiate such surface contacts. Adhesion is also the first step in the infectious process for pathogenic pseudomonads. Progress has been made in identifying adhesins of pseudomonads—these factors are variable in their specificity and strength of binding. In this chapter we will discuss the broad range of adhesins identified in different species of *Pseudomonas*.

2. *PSEUDOMONAS AERUGINOSA*

Pseudomonas aeruginosa is a human opportunistic pathogen posing a major threat to cystic fibrosis (CF) patients, as well as individuals with compromised immune systems, device implants and burns⁹⁰. The ability of *P. aeruginosa* to interact with the surfaces encountered in these diverse environments suggests that this opportunistic pathogen may have evolved a strategy whereby it elaborates either an impressive variety of adhesins, or alternatively, multifunctional

adhesins that allow adherence to structurally unrelated surfaces. As outlined in the following sections, it is becoming clear that *P. aeruginosa* has adopted both of these strategies, no doubt contributing to the success of this organism inside and outside of the host.

2.1. Adherence to Mucin

The human respiratory tract secretes a thick layer of mucus, which contains mucin⁴⁷. Mucins are glycoproteins with a wide spectrum of oligosaccharide structures and are a component of the mucociliary escalator system normally responsible for removing inhaled particles or microorganisms from the airway and lungs^{47, 78}. Hypersecretion of mucin is detrimental, as in chronic bronchitis and CF patients, in part because the additional binding sites provided for bacteria may potentially enhance infection⁴⁷. Mucin derived from CF patients has been shown to have alterations in carbohydrate composition and glycosylation of mucin glycopeptides compared to mucin from healthy individuals^{13, 16, 17, 32, 84, 85, 99}.

Viswanath and Ramphal coated silicon-treated 96-well plates with human tracheobronchial mucin (HTBM) from the lungs of healthy individuals and then quantitated the attachment of *P. aeruginosa* to this mucin⁹². The number of attached *P. aeruginosa* cells increased directly as the concentration of mucin. In contrast, similar studies performed with *Escherichia coli* and *Klebsiella pneumoniae* did not show increased bacterial attachment, suggesting that mucin attachment is not a general property of all bacteria, but specific to *P. aeruginosa*⁹². Ten clinical strains of *P. aeruginosa*, isolated from CF patients, readily attached to tracheobronchial mucin and porcine gastric mucin, however *E. coli* K-12, *Proteus mirabilis* and *Klebsiella aerogenes* displayed no such interaction⁵⁹. Interestingly, purified mucin from the CF lung can act as a chemoattractant for most *P. aeruginosa* strains, possibly contributing to the ability of these microbes to seek out and attach to mucin in the CF lung⁵⁹. These experiments were complemented by transmission electron microscopy experiments that directly visualized the interaction of *P. aeruginosa* with mucin from CF patients⁵⁹. The CF mucin aggregated and attached to the bacterial cell surface, but this interaction was not observed when CF mucin was incubated with *E. coli*, *P. mirabilis* and *K. aerogenes*, again suggesting that this is a property specific to *P. aeruginosa*. These data suggest that binding of the mucin-containing mucus layer by *P. aeruginosa* may be a step in colonization of the CF lung by this opportunistic pathogen.

The characterization of the moiety of mucin recognized by *P. aeruginosa* was undertaken by studying treatment regimens that disrupted bacterial attachment to mucin. Attachment is sensitive to periodate oxidation, which suggests the moiety recognized by the bacterial adhesin(s) is a carbohydrate.

Furthermore, addition of the soluble amino sugars *n*-acetylglucosamine and *n*-acetylneuraminic acid inhibited the adherence of both mucoid and non-mucoid *P. aeruginosa* strains to mucin⁹³. This result suggested that *n*-acetylglucosamine and *n*-acetylneuraminic acid, or closely related structure(s), may make up an important component of the binding site of tracheobronchial mucin, and thus are able to compete for bacterial cell binding with mucin.

Subsequent studies set out to further define the site of interaction between *P. aeruginosa* and mucin, and showed that *P. aeruginosa* specifically recognizes carbohydrates containing type 1 (Gal β 1-3GlcNAc) and type 2 (Gal β 1-4GlcNAc) disaccharide units⁶⁵. Type 1 and type 2 disaccharide units are part of the oligosaccharide backbone or peripheral decorations of mucin and may be possible binding sites for bacteria⁷³. *P. aeruginosa* adhesion to the disaccharides was tested in a microtiter adhesion assay, in which the wells were coated with the disaccharides, and by a thin-layer chromatography bacterial overlay assay⁶⁵. Strains lacking the sole type IV pilus produced by *P. aeruginosa* were generated in a virulent hyperpiliated strain (1244-NP) and a common laboratory strain (PAK-NP). 1244-NP attached to the type 1 and type 2 disaccharides at 2–3 times the number of the wild-type 1244 strain, while PAK-NP attached 2 fold higher than PAK to the type 2 disaccharide⁶⁵. Furthermore, two antibodies raised against type 2 disaccharide partially inhibited binding of both piliated and non-piliated strains. These data suggested that type 1 and type 2 disaccharides are recognized by *P. aeruginosa*, but in a pili-independent fashion⁶⁵. Although it is still unclear to what extent the type 1 and type 2 disaccharides are available for binding to *P. aeruginosa* *in vivo*, these studies showed that *P. aeruginosa* has the capacity to bind to polysaccharide components of mucin.

Ramphal and colleagues began investigating the bacterial adhesins that are required for attachment to purified mucin by first examining the affect of a type IV pilus mutant compared to its wild-type parental strain *P. aeruginosa* PAK⁶⁶. *P. aeruginosa* has a polar localized type IV pilus composed of a polymer of a single protein subunit, called pilin^{42, 98}. An *in vitro* mucin attachment system was developed utilizing purified HTBM to coat the wells of microtiter dishes. The pilin mutants attached to mucin to the same degree as wild-type cells⁶⁶. This study suggested that adhesins other than pili are necessary for attachment to mucin, a result in good agreement with the outcome of the binding studies with type 1 and type 2 disaccharides outlined above.

Simpson and colleagues screened for mutant strains blocked in the production of these mucin adhesins using two methods^{3, 80, 81}. The first method was a screen in a mutant lacking pili for derivatives that were defective in binding to mucin and to A549 epithelial cells. Their rationale for such an approach was based on previous work showing that pili only played a minor role in bacterial attachment to mucin, compared with their relatively large role in attachment to epithelial cells^{18, 66}. Three of the mutants have been identified, two map to the

fliO gene and one to the *fliF* gene^{3, 81}. Providing a wild-type copy of *fliO* in trans could complement the attachment defect of the *fliO* mutants and restore motility. Homologs of FliO are involved in the biosynthesis of flagella, and specifically, are thought to be involved in the export of flagellar proteins⁵⁴, although the exact role of this protein in flagellar biosynthesis has not yet been determined. The *fliF* gene was also determined to be important for attachment to mucin³. The *fliF* gene encodes the membrane and super-membrane ring of the flagellum and in *P. aeruginosa* it was found to be transcribed in the same direction as *fliE* and *fliG* genes, possibly as part of a single operon³. The *fliE* gene encodes for a basal body component of the flagella, while the *fliG* gene encodes for the switch component of the flagellum structure⁵³. The data suggest that the basal body structures may be important for the localization of an adhesin, or alternatively, that flagellar-mediated motility is required for adhesion³.

Ritchings *et al.* identified two additional loci required for mucin attachment, *fleS* and *fleR*⁷⁰. FleS and FleR comprise a two component regulatory system based on sequence similarity to other members of this family of proteins and regulate the biosynthesis of the motility machinery, and likely indirectly affect mucin adhesion⁷⁰.

To further address the role of the flagellum in adhesion to mucin, Simpson and colleagues generated pilin and flagellum double mutants⁸⁰. A mutation in *fliC*, the flagellin subunit protein, was introduced into a pilin mutant background and this double mutant, while nonmotile, was still able to adhere to mucin. Also, a mutation in *fliA*, a gene necessary for flagellin expression, was moved into a pilin mutant strain and this strain was also nonmotile, but remained capable of mucin adhesion⁸⁰. In contrast to the data presented above, these results suggest that neither the flagellum nor flagella-mediated motility is required for adhesion to mucin. It is difficult to reconcile why other components of the flagellum biosynthesis pathway and structural components of the flagellum, such as *fliO*, *fliF*, *fleR* and *fleS*, are apparently important for adhesin. One explanation for these contradictory results is that the flagella machinery, which has sequence similarity to type III secretion systems⁶², secretes an adhesin other than the flagellum.

The flagellar cap protein was also investigated for its role in adhesion. Arora *et al.* tested a polar chromosomal insertional mutation in the *fliD* gene of *P. aeruginosa* PAK-NP (this strain is also non-piliated) for attachment to mucin and found this mutant could no longer attach to mucin as well as the parental strain PAK-NP⁴. The *fliD* gene on a multicopy plasmid could restore mucin attachment and motility and purified FliD protein was able to inhibit binding of *P. aeruginosa* PAK-NP to human respiratory mucins⁴, demonstrating that this protein is important for bacterial-mucin interactions.

Further investigation has led to the report of two types of FliD proteins, type A in strain PAK and type B in strain PAO1^{2, 77}. The two proteins show

only 43% identity at the amino acid level and there is no immunological cross-reactivity between them determined by experiments performed with polyclonal antibodies, thus the binding characteristics of proteins may differ from each other². To determine the binding of the type A and type B variants to the glycoconjugate components of mucin, a microtiter dish was coated with these modified polysaccharides and then bacterial adherence was quantitated. As an alternative method, bacterial attachment to fluorescent glycoconjugates was measured in solution and the K_d was determined for this interaction⁷⁷. Type B FliD of PAO1 was shown to bind to glycoconjugates with Lewis x or sialyl-Lewis x determinants⁷⁸. Studies conducted on the mucin from patients chronically colonized by bacteria, found that sialyl-Lewis x determinants are overexpressed in these mucins²⁰. In contrast, the type A cap protein of *P. aeruginosa* PAK could not bind to glycoconjugates with the Lewis x, sialyl-Lewis-x, or sulfosialyl-Lewis x glycotypes⁷⁷. Lewis determinants are carbohydrate antigens present on human epithelial cell mucins as well as red blood cells. It is believed they function as receptors for lectins^{6, 31}. These results suggest that there is specificity between the *P. aeruginosa* species-specific flagellar-associated adhesins and mucin glycotypes.

Ramphal and colleagues also tested an *rpoN* mutant in their in vitro mucin attachment assay⁶⁶. An alternative sigma subunit of RNA polymerase, RpoN, has been shown to be required for pilin gene expression, flagella expression and motility and also affects the expression of other non-pilus adhesins used to attach to mucin as well as epithelial cells^{18, 66, 74}. The *rpoN* mutation resulted in a significant reduction of adherence to mucin⁶⁶. However, because of the pleiotropic nature of the *rpoN* mutation it is difficult to determine the relative roles of flagella, flagellar-mediated motility, and pili and/or non-pilus adhesions to this process. To further investigate the role of *rpoN* in attachment to mucin, Simpson and colleagues performed a mutagenesis of an *rpoN* mutant strain (*P. aeruginosa* N1G) and assayed the mutants for the restored ability to adhere to mucin and epithelial cells⁸⁰. The rationale underlying this screen was to identify derivatives of an *rpoN* mutant that had regained their ability to attach to mucins and to A549 epithelial cells. Two classes of mutants were identified in this screen. One class included two mutants that were isolated because of their increased attachment to mucin, however these two mutants do not have increased attachment to epithelial cells. A second class was represented by one mutant that attached to epithelial cells several fold higher than wild type and had a modest increase in attachment to mucin when compared to the *rpoN* mutant strain⁸⁰. The mutations responsible for these phenotypes still remain to be determined.

In summary, the role of the flagellum and flagellar-mediated motility for adherence is controversial. Complicating matters are data suggesting that the requirement for the flagellum and/or flagellar-mediated motility may vary

depending on the organism, surface encountered and the environmental signals perceived by the bacterium. Furthermore, some of the data presented above are in conflict, so it is difficult to make any broad conclusions as to the precise role of the flagellum and the flagellum secretion machinery in initial surface interactions on biotic and abiotic surfaces based on the currently available data.

Outer membrane proteins (OMP) have been shown to play a role in bacterial adhesion to a broad range of substrates. Carnoy and colleagues identified OMPs that appear to be involved in attachment to mucin. OMP preparations of two adhesive *P. aeruginosa* strains (1244-NP and PAK-NP), as well as *rpoN* mutants of these strains (the *rpoN* mutation renders these strains poorly adhesive) were resolved by SDS-PAGE, blotted, and subsequently probed with radiolabeled mucin and lactotransferrin to identify proteins with the ability to bind these glycoproteins¹⁴. Lactotransferrin, like mucin, is a glycoprotein secreted into human mucus, however it is N-linked as opposed to the O-linked mucin glycoprotein. Two OMPs derived from PAK-NP were identified that attached to mucin (but not to lactotransferrin) in this assay, while three such proteins were identified from 1244-NP OMP preparations. OMP preparations from the nonadhesive *rpoN* mutants of each strain produced similar mucin-binding proteins, however the signal detected was significantly weaker than that observed for the OMPs isolated from the parental strains¹⁴. These data suggest that both strains produced mucin-binding OMP that were under the control of the RpoN sigma factor. Tetramethylurea, which is thought to disrupt nonspecific, hydrophobic protein–mucin interactions, was added to blots probed with mucin¹⁴. No difference was observed between tetramethylurea treated and untreated blots suggesting there may be a specific interaction between the OMPs and mucin. Two additional OMPs from PAK-NP and 1244-NP were detected in the assay outlined above that bound both mucin and lactotransferrin¹⁴. These proteins may have two distinct binding sites, one for mucin and one for lactotransferrin, or one domain on the protein may recognize both glycoproteins. These data suggest that *P. aeruginosa* may express several adhesins in the OMP used for attachment to mucin as well as lactotransferrin. These OMPs have not yet been identified.

Studies performed by Reddy, identified a 16-kDa protein as an adhesin involved in the attachment of *P. aeruginosa* to HTBM⁶⁹. In this experiment HTBM was isolated and fractionated to obtain a high molecular weight fraction containing HTBM and linked proteins. The linked proteins were dissociated by reductive methylation and the remaining HTBM was used in an overlay binding assay with *P. aeruginosa* extracts. The 16-kDa *P. aeruginosa* protein identified by this binding assay is believed to interact with mucin via a protein–protein interaction because treatment of the *P. aeruginosa* extract with trypsin abolished binding⁶⁹. This protein remains to be identified. It is important to note that the extract of bacterial proteins tested in this assay may

contain cytoplasmic proteins as well as membrane proteins. If this protein is determined to be cytoplasmic then the results of these experiments could be interpreted in two different ways. First, there may be no physiologically relevant interaction between this 16-kDa cytoplasmic protein and HTBM, because this bacterial protein may never be found outside of the cell. Alternatively, it is formally possible that this protein could be secreted and therefore interact with HTBM outside of the bacterial cell. Further study of this 16-kDa protein is needed to determine its role, if any, in adhesion to mucin.

2.2. Adherence to Cell-Associated Mucin

Mucin is not only found secreted in mucus, but it is also found attached to epithelial cells. Respiratory epithelial cells abundantly express Muc1 mucin, and Lillehoj and colleagues determined that *P. aeruginosa* specifically binds to hamster Muc1 mucin^{51, 52}. They used Chinese hamster ovary (CHO) cells transfected with Muc1 cDNA or with an empty plasmid to test the ability of wild type, a *fliD* mutant strain, a pili mutant strain and a *fliC* mutant strain to attach to Muc1. The *fliC* mutant strain (FliC is the flagellin) was the only strain not able to attach to Muc1⁵². These results contradict results presented earlier, which found that FliD is important for attachment to mucin prepared from human sputum⁴. The same *fliD* nonmotile mutant strains were used in both studies, but it is possible that the Muc1 mucin has different binding sites than secreted mucin. Treatment of the CHO-Muc1 cells with purified flagellin disrupted binding of bacteria, as did treatment of bacteria with flagellin antiserum, although the motility of these strains after treatment with the antiserum was not tested⁵².

As described above adhesins important for binding epithelial cells and mucin have been identified, and further investigation has led to the identification of common receptors for bacterial binding on both of these substrates. *P. aeruginosa* has been shown to produce high levels of D-galactose and L-fucose binding lectins (PA-IL and PA-IIL)³⁴. Cell surface glycoconjugates and CF-derived mucins carry fucose as the terminal residue on complex oligosaccharide antigens of the Lewis A or Le^x series, and serve as binding sites for PA-IIL⁵⁸. The sugar affinities of the PA-IL and PA-IIL lectins have been determined and both have a high affinity for fucose³³. Mitchell and colleagues reported the high-resolution crystal structure of PA-IIL in complex with fucose and determined that the quaternary structure of PA-IIL is a tetramer⁵⁸. Two calcium-binding loops are present on PA-IIL and it is believed the fucose locks onto both calcium ions when this sugar binds to PA-IIL. These studies also found that Lewis A (or Le^a) active pentasaccharide (lacto-N-fucopentaose) is the most potent inhibitor of PA-IIL to human blood group H binding. This is significant because *P. aeruginosa* can also cause bacteremia

in immunocompromised patients and finding strategies to inhibit PA-III mediated binding may decrease systemic disease⁸³.

2.3. Pilus Adhesins

P. aeruginosa does not appear to exhibit any apparent tissue specificity, but infects tissues of different origins that have been previously damaged⁶⁴. Bacterial attachment to trypsinized human buccal cells has been shown to be an effective *in vitro* system for studying colonization^{27, 68, 74, 95}. The pili was shown to be an important structure for adherence of non-mucoid bacteria to epithelial cells, however, pili were found not to be an important adhesin of mucoid bacterial cells⁶⁸. There was some residual attachment of a *pil*⁻ non-mucoid strain, suggesting other adhesins are also involved in this interaction with epithelial cells⁶⁸. Furthermore, several investigators have demonstrated that the epithelial cell-binding domain of the *P. aeruginosa* PAK pilin structural protein lies in the C-terminal region^{11, 42, 49}.

The pilin may promote the interaction of bacterial cells with epithelial cells through contacts with glycosphingolipids on the surface of the host cells⁷⁵. Glycosphingolipids contain one or more monosaccharides linked to ceramide, the lipid moiety of the glycosphingolipid. Glycosphingolipids have been found in the plasma membrane of all eukaryotic cells, usually localized to the outer leaflet of the membrane. These molecules have been identified as important receptors for the binding of both bacterial cells and toxins⁹⁶. Krivan and colleagues determined that *P. aeruginosa* and *Burkholderia cepacia* CF isolates bound specifically to glycosphingolipids containing terminal or internal GalNAc β 1-4Gal sequences, such as asialoganglioside 1 (asialo-GM₁). Furthermore they determined that asialo-GM₁ occurs in substantial amounts in human lung tissue^{45, 46}. De Bentzmann and colleagues also found that asialo-GM₁ is present on both CF and non-CF regenerating cells²¹. Furthermore, an antibody to asialo-GM₁ effectively displaced *P. aeruginosa* from its binding site^{21, 41}. Saiman and Prince extended these studies and determined CF epithelial cells in primary culture contained superficial asialo-GM₁ on 12 % of cells compared to 2.9% of normal control cells⁷⁵. This increased asialo-GM₁ in CF cell lines suggest the presence of additional binding sites for the bacterium in the CF lung. Exogenous asialo-GM₁ was able to competitively inhibit *P. aeruginosa* adherence to epithelial cells, consistent with its role as a receptor. Glycosphingolipids were further characterized to determine the sites required for *P. aeruginosa* pili-mediated adherence⁷⁹. It was determined that both *P. aeruginosa* PAK and PAO strains bound to the GalNAc(1-4) β Gal sequence in asialo-GM₁ and asialo-GM₂, determined by solid-phase binding assays⁷⁹.

Gupta and colleagues demonstrated an interaction between partially purified pili (with reduced levels of LPS) and asialo-GM₁, and between LPS

and asialo-GM₁, using thin-layer chromatography³⁶. Taken together, these data strongly suggested that asialo-GM₁ could act as a bacterial receptor on the cell surface and that the bacteria used a combination of its pilus and/or LPS to mediate this interaction. However, it is not clear if the cell lines used in these experiments produced significant levels of mucin, or if mucin production would have affected the outcome of these studies.

Adherence of *P. aeruginosa* to endothelial cells has been studied to determine possible mechanisms of bacterial dissemination. *P. aeruginosa* strain PAK, two isogenic non-piliated strains, which carry a mutation in the structural pilin gene (PAK-NP) or a mutant in the regulatory *rpoN* gene (PAK-N1), were tested for attachment to human endothelial cells in primary culture⁶³. The pilated strain adhered in significantly higher numbers than either of the non-piliated strains. The fact that there was some attachment of the non-piliated strains led Plotkowski and colleagues to propose the possibility of other adhesins also playing a role in this interaction⁶³.

2.4. Non-Pilus Adhesins for Attachment to Eukaryotic Cells

Plotkowski and colleagues have studied the interaction of *P. aeruginosa* and laminin, a noncollagenous glycoprotein found in the basement membrane of epithelia⁶⁴. To determine if there was a link between the ability to bind to laminin and infectivity, isolated strains from asymptomatic carriers and strains from infected patients were compared for binding to laminin. No difference was observed for the binding between the bacteria isolated from infected patients and those isolated from asymptomatic carriers, which could mean that the adhesin responsible for binding laminin is encoded on the genome of many or most *P. aeruginosa* strains⁶⁴. Further studies found no difference in the binding of pilated and non-piliated strains to laminin. These results suggest a non-pili adhesin is responsible for binding laminin. Laminin binding was localized to the outermost layer of the bacterium by transmission immunoelectron microscopy⁶⁴. In these experiments bacteria were incubated successively with laminin, anti-laminin antibody and protein A-colloidal gold complex. The OMPs were separated on a polyacrylamide gel and probed with laminin, resulting in the identification of two bands, a 57- and a 59-kDa non-pilus adhesins. These putative non-pilus adhesins have not yet been identified. These results demonstrate the binding of *Pseudomonas* isolates to a component of the basement membrane of damaged cells utilizing an adhesin other than the pilus. Further study is needed to determine the role of these putative adhesins in vivo.

Porin F (OprF) has been identified as an adhesin for attachment to human alveolar A549 epithelial cells⁵. An isogenic mutant strain adhered 43% less than the wild-type strain in adhesion assays. A549 cells pretreated with

purified OprF also showed reduced bacterial binding. Recent studies by Yoon and colleagues indicated that OprF is also important for biofilm formation in anaerobic conditions⁹⁷.

In addition to cell surface proteins, there are several examples of secreted proteins, such as toxins that play a role in adhesion (e.g., pertussis toxin and alpha toxin)^{1, 12}. Baker and colleagues investigated the role of the secreted exoenzyme S in the adhesion of *P. aeruginosa* to epithelial cells and they found exoenzyme S to be localized to the cell surface by electron microscopy⁷. Both exoenzyme S and a monoclonal antibody to exoenzyme S are able to inhibit binding of *P. aeruginosa* to buccal cells. Exoenzyme S also is able to bind to glycosphingolipids, at a degree similar to that reported for intact bacteria⁷. Other bacteria and bacterial toxins such as cholera toxin bind to glycosphingolipids on epithelial cells³⁰. It appears from these studies that there may be two functions for some bacterial toxins, a role in host cell toxicity and a role in attachment.

Another site of *P. aeruginosa* infection is the human cornea which results in *Pseudomonas* keratitis. Alkaline protease, LPS, and pili play a role in adhesion of *P. aeruginosa* to human corneal epithelial cells³⁶⁻³⁸. *P. aeruginosa* was examined for attachment to human corneal epithelial proteins (HCEP) *in vitro*, and it was found that the outer core portion of LPS is critical for binding of the bacteria to HCEP³⁸. This binding was also observed for attachment of *P. aeruginosa* to sacrificed corneal epithelium, and binding was blocked in presence of antibodies directed against the outer core of LPS. Gupta and colleagues also identified LPS binding proteins present in total extract from HCEP. The HCEP proteins were run on an SDS-PAGE gel and transferred to a nitrocellulose membrane before probing with biotin-labeled LPS. Five major protein bands were observed, ranging in size from 18 to 66 kDa. Galectin-3, a soluble animal lectin, was found to be one of the LPS binding proteins on the epithelial cell. Western blotting with an anti-galectin antibody revealed that galectin is a component HCEP. The interaction between LPS and HCEP was further studied in a solid-phase binding assay, in which a 96-well plate was coated with HCEP and then incubated with LPS. The results suggest that the binding is a receptor-ligand type of interaction and the binding is specific and saturable. An *in vitro* binding assay demonstrated that antibodies directed against the outer core of LPS or galectin-3 were able to significantly inhibit bacterial binding.

Ramphal and Pier used the injured tracheal cell model system to test the ability of *P. aeruginosa* exopolysaccharide (EPS) to act as an adhesin⁶⁷. This model system may mimic some aspects of the environment found in the lungs of CF patients. Radiolabeled mucoid *P. aeruginosa* EPS attached better to injured tracheal cells than to normal tracheal cells. An antibody directed against the EPS inhibited the adherence of four out of five mucoid strains⁶⁷. This study suggests that mucoid *P. aeruginosa* strains are equipped to attach to injured cells by a different mechanism than that they use to attach to healthy cells.

Finally, Ramphal and colleagues studied two clinical *Pseudomonas* strains, which are capable of agglutinating erythrocytes (strains 1244 and CF613)⁶⁶. They generated *rpoN* mutations in each of these isolates and saw that this mutation had a minimal effect on the agglutination phenotype. This demonstrates the existence of yet another class of adhesins that are required for agglutination of erythrocytes and are distinct from adhesins needed for attachment to epithelial cells and mucin⁶⁶. The nature of these adhesins has not been investigated.

2.5. Adhesion to Plastic

The flagellum has been identified as playing an important role in the initial interaction of the bacterium and abiotic surfaces (both glass and plastic) by transposon mutagenesis and microscopy analysis in a flow system^{60, 76}. However, flow cell analysis by Klausen and colleagues in a citrate media (unlike the glucose-based media used in the previous experiments) did not find a role for flagella in initial attachment, instead a flagella mutant strain had an altered biofilm structure when compared to wild type⁴⁴. These data suggest that nutritional signals can influence the adhesion pathway employed.

Under conditions where flagella-mediated motility is required for adherence, it was postulated that the flagellum is needed for the bacteria to move toward the surface and to make initial surface interactions that may lead to attachment of the bacterial cell to the surface. The flagellum itself may also act as an adhesin, tethering the bacterium to the surface in a process referred to as reversible attachment. Whether the flagellum is needed for motility and/or adhesion has not yet been elucidated. The flagellum does not appear to be important after this initial interaction with the surface⁷⁶.

O'Toole and Kolter also studied pilin mutants and found there was no difference in initial contacts with the surface compared to wild-type cells⁶⁰. Further studies by Chiang and Burrows tested hyperpiliated *P. aeruginosa* in static attachment assays and the hyperpiliated cells were able to form dense biofilms even thicker than wild-type cells¹⁹. Vallet *et al.* analyzed the Tn5 insertion library of a non-piliated strain of *P. aeruginosa* to identify possible adhesins (or adhesin regulators) and identified the *cupA1-A5* genes⁸⁷. The *cupA* locus specifies components of a chaperone/usher pathway, thus may be involved in the transport of an adhesin to the cell surface⁸⁷.

2.6. Summary

There are several common themes that have emerged from the studies of *P. aeruginosa* adhesion. First, in a search for mutants defective for adhering to a wide range of surfaces (including mucin, epithelial cells and abiotic surfaces)

several investigators isolated components of the flagellar biosynthetic machinery. The FliD cap protein in particular has been implicated as a flagellar-associated adhesin although there is at least one report inconsistent with the role of FliD as an adhesin. Type IV pili seem to be a key player for attaching to epithelial cells, but play a less significant role in bacterial interactions with mucin and abiotic surfaces. Several OMP proteins have also been implicated as adhesions using biochemical approaches, but the identities of these proteins are for the large part unknown and no genetic studies have been performed to confirm their role in adherence. LPS may also act as an adhesin in some circumstances. The regulation of many of the factors mentioned above, with a few exceptions, has only been poorly studied.

3. ENVIRONMENTAL PSEUDOMONADS

Pseudomonads such as *Pseudomonas syringae*, *Pseudomonas fluorescens* and *Pseudomonas putida* are important environmental bacteria, but do not typically cause disease in humans. *P. syringae* is typically thought of as a plant pathogen causing necrosis of plant leaves⁴⁰, however disease is only found on specific host plants for the *P. syringae* pathovar, while this bacterium can colonize a wide range of non-host plants without causing disease⁸². *P. fluorescens* and *P. putida* are generally found in the rhizosphere and soil environment as plant growth promoting rhizobacteria (PGPR) and these organisms share a high degree of sequence similarity with each other (Nelson, 2003 #140; http://www.jgi.doe.gov/JGI_microbial/html/pseudomonas/pseudo_homepage.html). These PGPR can offer the plant protection through a variety of mechanisms such as production of antimicrobials, niche exclusion, sequestering available nutrients through the production of siderophores, and binding to a plant root leading to induced systemic response^{28, 50, 88}.

Environmental pseudomonads are typically thought to occupy several environmental niches, including growing as planktonic cells, as well as living attached to biotic surfaces and/or abiotic surfaces. The biotic environment can either be the rhizosphere, the area on and in close proximity of plant roots, or the phyllosphere, referring to the portion of the plant above the ground. These niches offer nutrients from the plant and are very competitive areas for bacterial colonization. Also present in the environment are abiotic surfaces such as sand and soil. In this section we will discuss the adhesins utilized by these organisms to colonize this wide diversity of surfaces.

DeFlaun and colleagues developed a sand column adhesion assay to test the ability of environmental isolates and laboratory strains for their ability to attach to sand²⁶. These investigators found that bacteria in log phase attached much better than stationary phase cells. They also noticed that cells grown in

minimal media demonstrated increased attachment compared to cells grown in rich media (LB broth). These results suggest that bacterial attachment to surfaces may be regulated by nutritional and environmental signals^{26, 43, 56}.

The pilus has been identified as an important adhesin in biotic and abiotic systems. For example, phage $\phi 6$ -specific pili enhances the initiation of bacterial colonization of bean plants by *P. syringae* pathovar *syringae*⁸². Two pathovars have been studied for their ability to attach to plant leaves, *P. syringae* pv. *phaseolicola* HB10Y binds specifically to the stomata of the host plant, while *P. syringae* pv. *syringae* attaches evenly over the leaf surface⁷¹. Although there were spatial differences in their attachment, Romantschuk *et al.* determined that there was pili-mediated adsorption of bacterial cells to host and non-host plant leaves for both pathovars⁷². Non-piliated mutants adhered 2–10 fold less than the wild-type cells piliated bacteria, while super-piliated strains attach to a greater extent than the wild type^{71, 72}. Thus the pili appear to be an important adhesin for different pathovars of *P. syringae*, although the pili (or pili-associated proteins) of each pathovar appear to confer specificity for binding to the leaf surface.

The pilus has also been identified as an adhesin used for the attachment of *P. fluorescens* to biotic and abiotic surfaces. Vesper investigated *P. fluorescens* isolates 13252 and 2–79 for production of pili in rich and low nutrient media and found that peritrichously distributed pili are present in the higher numbers in low nutrient broth compared to rich media (LB) for both isolates⁹¹. Isolate 2–79 formed two colony types on rich media, mucoid and non-mucoid. Vesper determined that the non-mucoid cells were highly piliated in contrast to the poorly piliated mucoid cells. The isolates were also tested for their ability to cause hemagglutination, which is a common trait for bacteria expressing pili⁹¹. The highly piliated cells caused hemagglutination of five blood cell types, while the mucoid bacteria were unable to hemagglutinate any of the blood cell types tested. The non-mucoid cells bind better to the hydrophobic abiotic surface polystyrene and the biotic surface of corn roots compared to the mucoid cells⁹¹. Although the pili may be responsible for the differences in hemagglutination and attachment between the mucoid and non-mucoid strains, it is important to remember that the OM of these two strains may be very different, thus there are possibly other adhesins involved in this interaction.

de Weger and colleagues studied O-antigenic side chain mutants of *P. fluorescens* strains WCS358 and WCS374, and found that loss of the O-antigenic side chain changed the cell surface hydrophobicity and cell surface charge, but did not affect adhesion to positive and negative charged, hydrophilic and lipophilic surfaces, and potato roots²⁵. Williams and Fletcher screened transposon mutants of *P. fluorescens* H2 for those mutants with altered attachment to hydrophobic polystyrene and water wettable polystyrene⁹⁴. Four mutants were chosen for further study based on their increased adherence

to the hydrophobic surface and decreased adherence to water wettable polystyrene. These four mutant strains also adhere better than wild-type cells to quartz sand in a column adherence assay. Southern analysis identified that three of the insertions were siblings, thus two different genetic loci are responsible for this phenotype⁹⁴. These loci have yet to be identified. Analysis of the OM of these mutants by polyacrylamide gel electrophoresis showed that the O-antigen on the LPS was either attenuated or absent. It was hypothesized that lack of the O-antigen exposed more lipid moiety, which in turn may be responsible for the observed increased attachment.

De Mot and Vanderleyden isolated a major outer membrane protein (MOMP) from the rhizosphere-derived *P. fluorescens* OE 28.3. The MOMP was found to be attached to the roots of wheat, barley, maize and sunflower seedlings²³. MOMP binding to these surfaces was determined by immersing the plant roots in a suspension of OM derived from *P. fluorescens* OE 28.3, washing the roots to remove any attached proteins and then extracting attached proteins and analyzing them by two-dimensional gel electrophoresis. The gene encoding this MOMP was identified and it has sequence similarity with the amino- and carboxyterminal parts of porin F (OprF) from *P. syringae* and *P. aeruginosa*²². As discussed previously, OprF plays a role in attachment of *P. aeruginosa* to epithelial cells and in biofilm formation. Thus OprF appears to be somewhat conserved in pseudomonads in its role as an adhesin.

We have identified a large OMP (LapA) and ATP-binding cassette (ABC) transporter in *P. fluorescens* WCS365 that are important for adhesion to abiotic surfaces³⁹. Attachment of pseudomonads to an abiotic surface is thought to be a two-step process. Reversible attachment involves contact of the pole of the cell with the surface—this interaction is relatively weak and can be easily disrupted. Eventually the cell attaches by its long axis, so-called irreversible attachment, in which bacteria are very firmly attached to the surface^{48, 57, 100}. Experiments carried out in a flow cell system suggested that the ABC transporter and the LapA OMP are important for the transition from reversible to irreversible attachment. The LapA protein has a predicted molecular weight of ~880 kDa encoded by a predicted open reading frame of ~26 kb. This protein has also been identified in *P. putida* as an adhesin for attachment to corn seeds²⁹. Sequence analysis of published *Pseudomonas* genomes identified homologs of the LapA protein in *P. fluorescens* PfO1 and *P. putida*, but not in *P. aeruginosa* or *P. syringae*³⁹. It is possible that PGPR strains utilize an adhesin for abiotic surfaces not utilized by plant and human pathogenic *Pseudomonas*.

Epsinosa-Urgel and colleagues identified seven additional genes important for the biocontrol strain *P. putida* KT2440 to attach to corn seeds²⁹. Two of these mutants also had an adhesion-deficient phenotype on abiotic surfaces (glass and different plastics). Three of the mutants had significant sequence

similarity to genes of known function: a hemolysin, a peptide transporter, and a potential multidrug efflux pump. Of the four remaining transposon mutants one has limited similarity to a surface protein while the other three remaining genes are of unknown function²⁹.

Agglutination activity was correlated with the ability of bacteria to colonize roots, and may also correlate with bacterium-crop specificity^{10, 35, 89}. Buell and Anderson, identified a Tn5 transposon mutant of *Pputida* which prevented agglutination of the bacteria by bean root wash⁸. The *aggA* gene (1,356 bp open reading frame encoding a predicted 50-kDa protein) was found to be responsible for this phenotype. The AggA mutant strain was decreased in its ability to attach to bean roots ~9 fold compared to the wild type⁸. The expression of the *aggA* locus was monitored *in planta* by detection of the reporter gene *xylE*. The *aggA* locus promoter is active in *P. putida* cells removed from the bean root at 48 and 72 hr. Southern analysis of environmental pseudomonads found that the presence of *aggA* is limited to rhizosphere pseudomonads that express the agglutination phenotype⁹. We have identified the homolog of *aggA* in *P. fluorescens* WCS365 (*lapE*), and we predict based on sequence analysis and our experimental work that the encoded protein is part of the ABC transporter required for secretion of the LapA protein to the cell surface³⁹. In a *lapE/aggA* mutant strain, LapA is unable to reach the exterior of the cell implicating the involvement of LapE/AggA in the transport of LapA. It is possible that incorrect localization of a LapA homolog (and not the LapE/AggE protein *per se*) causes the observed agglutination phenotype in *P. putida*, however a mutant strain lacking LapA has not been tested in this agglutination assay.

Pseudomonas fluorescens PfO-1 was found to tightly adhere to sand using the sand column assay mentioned above, thus this strain was used for a transposon mutagenesis to identify the genes and proteins important for adhesion. A mutant was identified (PfO-5), which retained only 40–50% of the adherence ability of the wild-type strain²⁶. This mutant lacks a 34-kDa MOMP present in the wild type OM. Furthermore, transmission electron microscopy showed that this strain lacked the polar flagellum produced by the wild-type strain. This 34-kDa OM-localized protein may be the flagellin subunit, or alternatively may play a role in stabilizing or regulating the flagellin²⁶. Earlier studies by de Weger and colleagues found that motility was required for colonization of potato by *P. fluorescens* WCS374. They examined four flagella-less mutants and found that they attached as well as wild type on the highest part of the root, but they attached 3–4 times less than wild type at the deepest section of the root (closest to the root tip)²⁴. These results suggest that motility is required for the bacterium to move down the plant root and colonize the root tip.

Studies from the Levy lab focused on characterization of *adnA*, which is a transcription activator that has been shown to regulate a number of genes,

including those required for flagellar biosynthesis¹⁵. The flagellum is apparently neither essential for adhesion to sand nor for biofilm formation by *P. fluorescens* PfO1 under these conditions, therefore other genes activated by AdnA likely play a role in attachment^{15, 55}. O'Toole and Kolter showed that flagellar motility was important for early attachment to abiotic surfaces, but only under certain environmental conditions. That is, bacteria grown in minimal medium supplemented with glucose required a functional flagellum to adhere. This requirement could be bypassed by growing the nonmotile strains with citrate as the sole carbon and energy source⁶¹. These data suggest that there may be multiple, environmentally regulated pathways for adhesion to abiotic surfaces. In the case of biotic surfaces, Turnbull and colleagues studied the attachment of motile and nonmotile strains of *P. putida* PaW8 to sterile wheat roots. They determined that the nonmotile cells displayed decreased attachment in both competitive and noncompetitive attachment assays when compared to wild type⁸⁶.

4. SUMMARY

Pseudomonads are found in environments ranging from the human body to plant leaves to soil, yet there are common features that are required for attachment in all of these environments. Numerous studies have identified motility as an important factor for initial attachment events, but whether flagellar-mediated motility is required for attachment, the flagellum acts as an adhesin, or the flagellar machinery secretes one or more adhesins still remains to be determined in most systems. For *P. aeruginosa*, type IV pili have been found to play a central role in the attachment to epithelial cells, but are apparently less important for adherence to mucin and abiotic surfaces. Pili may also play a minor role as an adhesion for environmental pseudomonads, but much less work has been done addressing these questions in comparison to *P. aeruginosa*. LPS may also serve as an adhesin or influence the extent to which OMPs are available to participate in adherence.

Several OMPs have been identified as adhesins for biotic and abiotic surfaces. The approaches taken to date to identify such factors are largely biochemical. One such OMP, OprF, appears to be required for attachment by different pseudomonads, suggesting that this surface protein may be utilized in a variety of environments. However, the identity of most of these OMPs remains to be revealed, and their role in adhesion should be confirmed by disrupting the genes coding for these proteins and subjecting these mutants to a battery of attachment assays.

With a few exceptions, the regulation of these adhesins is an open area of investigation. Studies of pathogens and environmental pseudomonads suggest that nutritional status may be linked to adhesin production, prompting the

question of how these adhesins are regulated. Pseudomonads are superbly adapted organisms that display incredible metabolic diversity. Would it be so surprising that they have a repertoire of adhesions that matches their metabolic flexibility? And if such a collection of adhesins does exist, it is likely that their expression is tuned to particular environments. It is quite possible that “the adhesin” does not exist—there is no compelling evidence for such a universal adherence molecule. In contrast, attachment to a surface by pseudomonads is likely a multifactorial process. Despite the many studies performed examining bacterial attachment to surfaces, there is still a great deal to be learned regarding the mechanisms utilized by pseudomonads to adhere to the surfaces they encounter.

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FIMBRIAL GENES IN *PSEUDOMONAS* *AERUGINOSA* AND *PSEUDOMONAS* *PUTIDA*

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1. INTRODUCTION

Bacterial interactions with the environment or with other cells, sometimes mobility, are frequently associated with the function of extracellular appendages known as pili or fimbriae. The future of a host–bacterium interaction depends on the relationships that will be established. This interaction could be beneficial, or neutral, but turn out to pathogenesis when the interaction favours the targeting of bacterial factors, which results in damage to the host. In this respect, pili should be considered as key virulence factors in pathogenic bacteria. Pili are fibrous structures that are expressed on the surface of gram-negative bacteria. These complex structures usually result from the packing of a pilin, or fimbrial, subunit. Pili have been grouped on the basis of the amino acid sequence of pilin and on their assembly mechanism.

Type IV pili (TFP) were known for a long time as the only kind of pili found at the *Pseudomonas aeruginosa* cell surface⁵¹. Recently, many genes encoding other putative extracellular structures have been identified in the

P. aeruginosa genome⁷⁰. We mainly identified three gene clusters encoding chaperone-usher pathways that seem to play an important role in *P. aeruginosa* adhesion processes⁷³. Chaperone-usher pathways are found in many gram-negative bacteria, and allow the assembly of at least 40 architecturally different adhesive structures⁶⁹. The archetype of the chaperone-usher pathway is represented by the two systems acting in the assembly of the type P (pyelonephritis associated pili) and type I pili in *Escherichia coli*.

In this chapter, we will start with a description of TFP and then focus on the description of the novel chaperone-usher pathways that have been discovered in *Pseudomonas* species. Moreover, functional analysis of the *P. aeruginosa* genome revealed the existence of pili-like structures, such as the “tight adherence” system, which has been best described in *Actinobacillus actinomycetem-comitans*⁴⁰. We will thus give some details about these new fimbrial structures in *P. aeruginosa*.

2. TYPE IV PILI

Type IV pili are 5- to 7-nm thick rod-like fibres of variable length up to several micrometre⁵⁰. They are relatively flexible and usually located at the pole on the surface of bacterial cells. These structures have been extensively described in the case of many gram-negative bacteria, including *P. aeruginosa*, which will be used as a model in this chapter. The *P. aeruginosa* TFP have been shown to be essential components for attachment to plastic surfaces, to other bacterial cells or to eukaryotic cells. The attachment of pili to inert surfaces seems to occur via non-specific adhesion. The receptors for TFP at the surface of epithelial cells have been shown in many instances to be the carbohydrate moiety of the glycosphingolipids asialo-GM1 and asialo-GM2³⁰. In *P. aeruginosa*, the adhesion is mediated by a pilin C-terminal disulfide-bonded region, which is exposed at the tip of the pilus but otherwise buried within the filament⁵⁰. In *Neisseria* species, binding to epithelial cells requires an additional protein, PilC, which appears to function as a pilus tip adhesin⁵⁴. A homologue of PilC, PilY1, is also found in *P. aeruginosa*, but no evidence about a similar function has been provided³. Pili from different origins may have binding specificity for different type of cells. The attachment specificity is linked to tip-exposed binding sites and tip-associated adhesins, but also to structural features of the pilin itself. Moreover, TFP are required for the construction of sessile bacterial communities named “biofilm”⁵⁷. Attachment to human tissues and biofilm formation are two main traits of bacterial pathogenesis.

The TFP are not only required for microbial adhesion but are involved in bacterial movement based on pili retraction. These movements have been described as twitching motility for *Pseudomonas* or *Neisseria* species or social

gliding motility in *Myxococcus xanthus*⁷⁴. In *P. aeruginosa*, mutants incapable of twitching motility produce smooth domed colonies on agar plates, whereas wild-type colonies form flat, spreading colonies with characteristic rough appearance and a small peripheral twitching zone consisting of a thin layer of cells. Twitching motility is a means of rapid colonisation of new surfaces under conditions of high nutrient availability, whereas it may bring cells together in biofilms or fruiting bodies under conditions of nutrient depletion. Twitching and gliding motility are social behaviours since an isolated bacterium is unable to move. It was shown, in particular for *M. xanthus*, that the polarly located Tgl lipoprotein is able to stimulate, upon contact, the formation of TFP in the neighbouring cells⁷⁵. The Tgl homologue in *P. aeruginosa* is called PilF⁷⁶. Finally, TFP have been described as essential components for the development of natural competence and DNA uptake, like in *Neisseria* species or *Pseudomonas stutzeri*²⁷.

2.1. Molecular Mechanisms of Type IV Pili Assembly

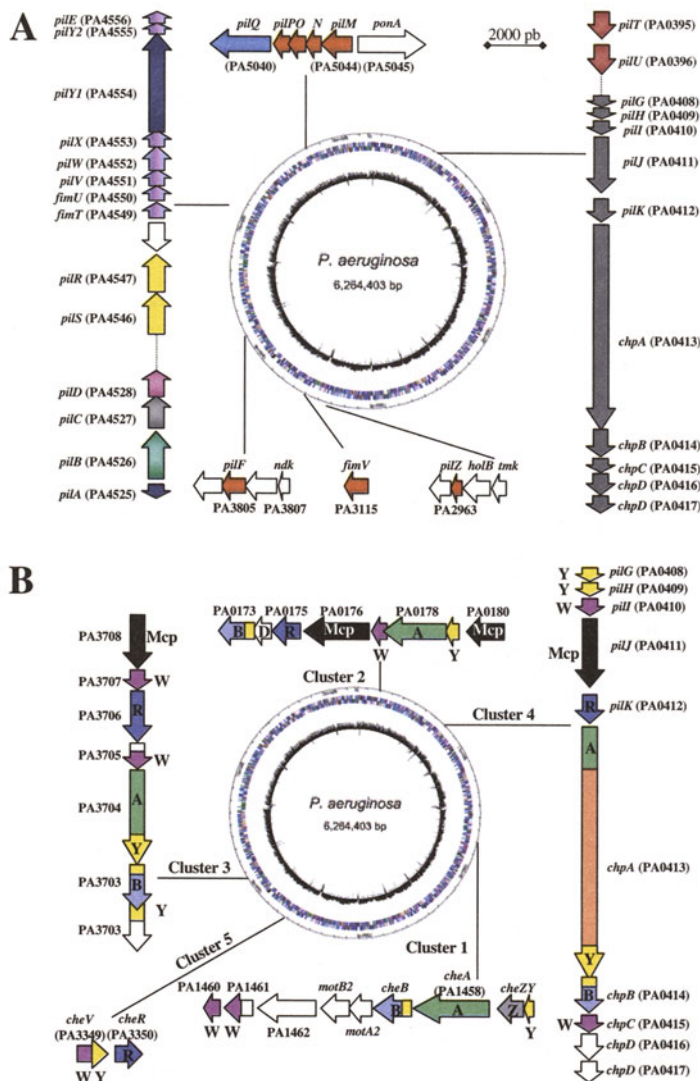
The TFP are composed from thousands copies of a single small protein subunit, usually termed PilA or pilin⁵⁰. Pilins are 140–160 amino acids in length with an extremely conserved N-terminal sequence (Table 1). The sequence consists of a basic leader peptide and a highly hydrophobic domain. In the course of assembly, the leader peptide is cleaved and the resulting N-terminal residue is methylated. Both cleavage and methylation are achieved by the bifunctional prepilin leader peptidase, PilD⁷¹. The cleavage site is invariably preceded by a glycine residue and followed by a hydrophobic residue, most frequently a phenylalanine, which is methylated once the cleavage is achieved (Table 1). One additional characteristic feature of the pilins is the presence of a glutamate residue within the hydrophobic stretch, at position +5 after the cleavage site (Table 1). It was shown that the glutamate residue is essential for assembly and methylation but not for cleavage⁵⁹.

Genetic analysis has revealed in *P. aeruginosa* the existence of other genes encoding pilin-like proteins. There are at least six of them (Table 1), with PilE, PilV, PilW, PilX, FimT and FimU that all contain the canonical N-terminal sequence and that are required for pili assembly (except for FimT which can substitute for FimU)⁵⁰. These minor pilins seem to be exclusively located in the cell membrane fraction suggesting that they may form the basis of the fibre structure. The PilX subunit is lacking the normally obligate E+5 (Table 1) and may function as an anchor or terminator of the pilus³. The *Neisseria pilC* homologue, *pilY1*, and another gene encoding a small protein, *pilY2*, are both located in a cluster encoding minor pilins in *P. aeruginosa*, between the *pilX* and *pilE* genes (Figure 1A).

Table 1. Pilins and pseudopilins in *P. aeruginosa* and *P. putida*.

Protein name ^a	Number ^b	Length (aa) ^c	N-terminal sequence ^d	Function
<i>P. aeruginosa</i> PAO1				
PilA	PA4525	149	GFTL IELMIVVAI	Major type IV pili prepilin
PilE	PA4556	141	GFTL LEMVVVVAV	Minor type IV pili prepilin
PilV	PA4551	185	GF SMIEVLVALLL	Minor type IV pili prepilin
PilW	PA4552	274	GL SMIELLVALAI	Minor type IV pili prepilin
PilX	PA4553	195	GATL VIALAILVI	Minor type IV pili prepilin
FimT	PA4549	169	ALT TELLFALVL	Minor type IV pili prepilin
FimU	PA4550	168	GFTL IELLIHVVL	Minor type IV pili prepilin
XcpT	PA3101	148	GFTL IEIMVVVVI	Type II secretion pseudopilin
XcpU	PA3100	172	GFTL IELMVMVMI	Type II secretion pseudopilin
XcpV	PA3099	129	GFTL LEVLVALAI	Type II secretion pseudopilin
XcpW	PA3098	237	GFTL LELLIAIAI	Type II secretion pseudopilin
XcpX	PA3097	333	GVAL ITVLLVVAV	Type II secretion pseudopilin
HxcT	PA0681	149	GFTL IEIMVVVVI	Type II secretion pseudopilin
HxcU	PA0678	149	GFTL IELMVVLVI	Type II secretion pseudopilin
HxcV	PA0680	124	GFTL IEVLVALAI	Type II secretion pseudopilin
HxcW	PA0677	213	GFTL IEVMVAIML	Type II secretion pseudopilin
HxcX	PA0682	321	GMA ISALLIVTV	Type II secretion pseudopilin
HplT	PA2675	144	GFTL LEMIVVLVI	Unknown
HplU	PA2674	136	AFTL LELLVVLVI	Unknown
HplV	PA2673	141	GFTL LEAVVALTL	Unknown
HplW	PA2672	196	AFTL LEMIVVLLV	Unknown
HplX	PA2671	359	GFV LVGVVWFLAI	Unknown
<i>P. putida</i> KT2440				
PilA	PP0634	136	GITL IELMIVVAI	Putative type IV pili prepilin
FimT	PP0607	160	GV TLIQMLSALAV	Putative minor type IV pili prepilin
	PP0609	214	GF GLVELMLALTI	Putative minor type IV pili prepilin
	PP0610	154	GV VLLALVLSVL	Putative minor type IV pili prepilin
PilE	PP0611	129	GL SLIELLIVLAV	Putative minor type IV pili prepilin
XcpT	PP1049	151	GFTL MEIMVVIFI	Putative type II secretion pseudopilin
XcpU	PP1050	143	GF SLIELLVVLAI	Putative type II secretion pseudopilin
XcpV	PP1051	135	GFTL LEVTVALAI	Putative type II secretion pseudopilin
XcpW	PP1052	196	GL TLIELMVALAL	Putative type II secretion pseudopilin
XcpX	PP1042	321	GA ALLMVMVVLAM	Putative type II secretion pseudopilin
XcmT1	PP3423	169	GFTL LELLVVLVV	Putative type IIb secretion pseudopilin
XcmX	PP3475	176	GFTY LGVLLLIAV	Putative type IIb secretion pseudopilin
XcmT3	PP3476	128	GFTL IELLVMAI	Putative type IIb secretion pseudopilin
XcmT2	PP3477	159	GF SLIEVVLTLAL	Putative type IIb secretion pseudopilin

^aThe name of the protein and the^bNumber are according to the gene annotation found at <http://www.pseudomonas.com> and <http://www.tigr.org/tigr-scripts/CMR2>.^cThe length of the protein is given in amino acids (aa).^dThe N-terminal sequence includes the conserved cleavage site by the prepilin peptidase. The canonical glycine (G) and phenylalanine (F) at position -1 and +1, respectively, are indicated in bold, as well as the glutamate (E) at position +5.



Assembly of the pilin subunit into a fibre requires, in addition to PilD, a traffic ATPase, PilB⁵⁶, a polytopic inner membrane protein PilC (PilF in *Neisseria*) and a multimeric outer membrane protein, the PilQ secretin⁴⁸, (see Chapter 26 of this volume). This last protein forms the gated channel into the outer membrane whose internal cavity diameter, 53 Å, closely matches that of the diameter of the corresponding pilus, 52 Å⁶. As we will see later, retraction of the pilus involves two additional traffic ATPases, PilT and PilU^{78, 79}. It should be noted that traffic ATPases and/or secretins belong to a superfamily of proteins that are widely involved in transport processes such as pili biogenesis, protein secretion, DNA uptake in gram-positive and gram-negative bacteria or filamentous phage extrusion (see Chapter 25 in Volume 1 and Chapter 17 in Volume 3).

Other genes are required for pili assembly (Figure 1A). The *pilQ* gene is located in an operon including *pilMNOPQ*⁴⁷. PilM contains motifs conserved in the ATP-binding domain of actin and shows homology with the rod-shape determining protein, MreB, and the cell division protein FtsA, from *E. coli*. This suggests that TFP assembly may be linked to cell shape and possibly directed through PilM to the cell pole. PilP is a small lipoprotein that is responsible for stabilising formation of PilQ multimers. PilN and PilO are proteins with N-terminal membrane anchor.

The product of the *fimV* gene is also required for pilus assembly and twitching motility⁶⁶. It is a highly acidic protein with a putative peptidoglycan-binding domain. Interestingly, overproduction of FimV causes dramatic elongation of the cells, which thus links its action to the cell cycle. Another link with the cell cycle is related to the role of PilZ², whose gene expression is transcriptionally coupled to *holB*, encoding the δ subunit of DNA polymerase III.

A large number of the proteins involved in TFP biogenesis have homologues that are required in other biological systems for transport of macromolecules and macromolecular complexes. These proteins are essentially the pilin subunits, the prepilin peptidase (PilD), the traffic ATPase (PilB), the polytopic inner membrane protein homologue of PilC, the secretin (PilQ) and its helper lipoprotein (PilP). The biological processes concerned are mainly protein secretion (see Chapter 26) and DNA uptake (no secretin in case of gram-positive bacteria), and to some extent filamentous phage extrusion, which only requires the secretin among the set of components previously described. These components appear to form similar complexes at the cell surface, which have a common evolutionary origin and core architecture, but which have thus been adapted to different functions.

2.2. The Structure of Type IV Pilin

The TFP structure was originally solved with *Neisseria gonorrhoeae* MS11 pilin⁵⁸, but recent structures of *P. aeruginosa* pilin from strain PAK³²,

and strain K122-4⁴³, showed that this general structure is conserved albeit with some variations. The thousands copies of pilin composing the pilus are arranged in a helical conformation with five subunits per turn and a pitch of 41 Å, resulting in a fibre with a 62 Å outer diameter. The pilin subunits within the fibre adopt a highly asymmetrical three-dimensional α - β roll fold. The conserved N-terminal hydrophobic domain will form the core of the pilus fibre. The core axis is formed by contact between the hydrophobic α -helices, which provide mechanical strength and flexibility. The α -helix core is wrapped by β -strands on which anchors the structurally variable globular head. The central and carboxy-terminal two thirds of the pilin subunit are relatively hydrophilic and contain the major sites of structural and antigenic variation. Thus, the outside part of the fibre contributes to surface variation for specificity of pilus function in antigenicity and adhesion. This variation also defines the serogroups and serotypes of the organism. It should be noted that the *Neisseria* and *Pseudomonas* models differ in the sense that the model presented for *P. aeruginosa* is a left-handed helix (dictated by the electrostatic surfaces) as opposed to a right-handed helix proposed for *Neisseria*. Finally, it is proposed that the C-terminal disulfide-bonded loop is part of the receptor-binding site. Since the adhesive properties of TFP are located at the tip of the pilus, it is suggested that the binding site would be exposed in the pilin monomer present at the pilus tip whereas the others are apparently embedded into the fibre during assembly³².

The crystal structure of the TFP (TcpA) from the *Vibrio cholerae* toxin-coregulated pilus (TCP) has also been solved¹⁴. In contrast to pilin subunits from *P. aeruginosa* and *Neisseria* that belong to the class a, the *V. cholerae* pilin belongs to the class b of TFP (see Section 2.6). The TcpA structure revealed conserved architectural features, but also key differences with class a pilin, such as the connectivity of the β sheet. The pilin subunits within the fibre are also differently organised, with three strands that form a helical twist around the fibre axis and have an axial separation of 45 Å¹³.

2.3. Retraction and Type IV Pili-Dependent Cellular Motility

The motive force for twitching motility is pili retraction. The retractile properties of TFP were originally suspected based on an observation by Bradley, who describes pilus-specific phage particles, which initially attach to pili before being brought at the bacterial cell surface¹⁰. More direct evidences have now been obtained. Recently, Skerker and Berg labelled the TFP of *P. aeruginosa* with an amino-specific Cy3 fluorescent dye and visualised them on a quartz slide. In this way they were able to directly observe the extension–retraction of

the fibres⁶⁷. Similarly, Merz and collaborators used laser tweezers to show that *N. gonorrhoeae* pili retract⁵³.

The reversible process of assembly–disassembly of the TFP fibres is linked to the function of two antagonistic ATPases, PilB and PilT. Both proteins belong to the traffic ATPases superfamily⁶⁰. A third traffic ATPase, PilU, is found in *P. aeruginosa*, *P. stutzeri* or *N. gonorrhoeae* that seems to co-operate to enhance, but is not absolutely required for, the function of PilT⁷⁹. PilB is required for assembly, whereas PilT is compulsory for disassembly. Indeed, a *P. aeruginosa pilT* mutant is covered with pili but due to the lack of retraction is completely unable to demonstrate any twitching motility properties. The rate of disassembly by PilT has been estimated to occur at around 1,000 pilin subunits/s⁵³. In a mutant that is also lacking the PilQ secretin (export pore is thus blocked), the hyperpiliation phenotype is replaced by an in-growth of pili and protrusion of membranes, with cells that become very sick. This phenomenon has nicely been shown in the group of Michael Koomey when studying type IV piliation in *N. gonorrhoeae*⁸⁰. Because the filament is a helical polymer, assembly–disassembly may occur upon filament rotation as a consequence of pilin insertion or removal from a fixed point. The assembly–disassembly process must occur from the base, as the hydrophobic α -helical core of the TFP is too tightly packed to allow macromolecular transport through the pilus. Finally, the combination of adhesion and retraction of the TFP fibres explain why this type of motility is well suited for moving on solid surfaces, whereas flagellum-dependent mobility works well in aquatic environment.

2.4. Twitching Motility and Chemotaxis

Apart for those genes involved in pili biogenesis, other genes are involved in transcriptional regulatory and chemosensory pathways that control expression and activity of the system. In total, some 40 genes have been identified whose product is required for twitching motility (Figure 1).

Transcription of *pilA* in *P. aeruginosa* is tightly controlled by a classical RpoN-dependent, two component sensor-regulator pair, PilS/PilR^{9, 33}. The PilS sensor is a transmembrane protein located at the pole of the cell for which the cognate signal has not been identified⁸. Twitching motility is also controlled via an atypical sensor-regulator pair FimS/AlgR⁷⁷ (see Chapter 9 in Volume 2).

The chemosensory system that controls pili-dependent motility in *P. aeruginosa* is similar, but more complex than, the chemotactic system that controls flagellar rotation in swimming motility (*che*) in several bacteria. The Che system operates via methyl-accepting chemotaxis proteins (MCPs), which

can induce autophosphorylation of a central histidine kinase (CheA), which in turn phosphorylates a response regulator (CheY) that reverses the direction of rotation of the flagellar motor. Other proteins, such as CheW, CheR, CheB and CheZ, act as modulators between MCPs and CheA, with for example CheB and CheR having methylase/methylesterase activities. *P. aeruginosa* genes encoding Che homologues that are involved in swimming motility have been previously identified (Figure 1B)^{25, 42, 49}. However, regulatory proteins involved in pili-dependent motility are distinct, and are located within the *chp* gene cluster (Figure 1B)^{15, 16}. This chemosensory pathway includes PilI and PilG, which are CheW and MCP homologues, respectively. The *chp* gene cluster also contains three CheY-like response receiver domains (Figure 1B). Two are on the PilG and PilH proteins, the third one at the C-terminus of the highly complex CheA homologue, ChpA. ChpA appears to be the most sophisticated signal transduction protein yet defined in nature. Indeed, in addition to the CheY domain, ChpA contains seven Hpt domains (see Chapter 9 in Volume 2), which suggests that this protein is receiving and integrating multiple signals and is part of a larger sensory cascade.

2.5. Type IV Pili-like Genes in the *P. aeruginosa* and *P. putida* Genomes

In addition to the pilin and pilin-like genes that have been found to be involved in type II protein secretion (see Chapter 26) and TFP biogenesis (this chapter), analysis of the *P. aeruginosa* genome sequence revealed additional such genes whose function has not yet been elucidated (Table 1). Indeed, a cluster of genes containing five putative pilin-like genes (PA2671-2675) has been identified. These genes were tentatively annotated *hpl* for homologous to *pil* genes (<http://www.pseudomonas.com>). The encoded proteins have all the canonical N-terminal sequence, including PA2671, which is lacking the glutamate at position +5 and is thus a PilX homologue (HplX). PA2672 and PA2674 (tentatively HplW and HplU) have an alanine residue instead of the conserved glycine residue at position -1, which is found in PA2673 and PA2675 (tentatively HplV and HplT). Interestingly, upstream of the pilin-like genes, two genes, PA2676 and PA2677, are encoding a polytopic membrane protein homologue to PilC (tentatively HplS) and a traffic ATPase (tentatively HplR), respectively. This suggests that HplR and HplS may be involved in the assembly of a pilus-like structure containing one or more of the pilin-like proteins. The composition of this gene cluster suggests that its function will probably not be related to type II secretion (see Chapter 26) but may be required for example in DNA uptake. The *comG* clusters of gram-positive bacteria, which are required for development of natural competence, are very similar to the *hpl*

cluster, and more interestingly it is not rare to find ComG pilin-like with an alanine residue at position -1, such as ComGC and ComGD from *Streptococcus pneumoniae*. The lack in the *hpl* cluster of secretin-encoding gene to allow extrusion of the pilus structure, and the fact that *P. aeruginosa* is not naturally competent for DNA uptake, call for experimental data that could confirm this hypothesis.

The presence of TFP-like genes in the *P. putida* KT2440 genome sequence⁵⁵ is reported in Table 1. The PilA homologue is encoded by the gene annotated PP0634, transcribed in opposite direction with respect to PP0633 and PP0632 encoding PilC and PilD homologues, respectively. However, the *pilB* gene is lacking in this cluster but may be corresponding to the isolated PP5190 gene, which encodes a putative traffic ATPase. Another traffic ATPase, similar to the *P. aeruginosa* PilT protein, is encoded by the PP5093 gene. Like in *P. aeruginosa*, the *pilQ* gene (PP5080), encoding the secretin, is found in a gene cluster encoding PilMNOP homologues (PP5081-PP5083). In this case, the PP5081 gene encodes a protein with the N-terminus showing similarity to PilO and the C-terminus to PilP. Just like in the *P. aeruginosa* cluster³⁴, the upstream gene (PP5084) is encoding a penicillin-binding protein. Additional genes encoding so-called minor pseudopilins are found in a cluster containing PP0607 (*fimT* homologue), PP0609 and PP0611 (*pilE* homologue). Additional pilin-like genes are found, most notably with PP3423, which is located next to a gene encoding a PilC homologue (PP3424) and a gene encoding a lytic transglycosylase (PP3422). Finally, PP3476 is an *xcpT* gene homologue found between putative *xcpX* and another *xcpT* homologues, PP3477 and PP3475, respectively (Table 1). Upstream of these genes, a secretin encoding gene (PP3478) and a traffic ATPase encoding gene (PP3483) are also found, whereas downstream, curli assembly-like gene homologues are found, notably *csgEFG* (PP3474-PP3472) (see Section 6). The set of genes, including PP3423-PP3424 and PP3475-pp3483, have been recently identified in *P. putida* GB-1. In this organism, these genes have been called *xcm* and are involved in the secretion of a manganese-oxidizing factor (see Chapter 26). The Xcm system is slightly deviating from the classical type II system. Accordingly, it has been classified in a subgroup named type IIb. Finally, the pilin-like genes that take part in the type II protein secretion process (see Chapter 25) are found in the large *xcp* gene cluster PP1043-PP1055 (Table 1).

2.6. The Class b of Type IV Pili

The type IV fimbriae called the bundle-forming pili (BFP) belongs to the class b of TFP and are found in enteropathogenic *E. coli* (EPEC) that are a leading cause of severe diarrhoea in infants throughout the developing world²¹. These fimbriae have a tendency to aggregate into rope-like bundles

and are responsible for the first stage of interaction between EPEC and host cells. The *bfp* cluster contains 14 genes whose products are sufficient for the biogenesis of BFP in a heterologous *E. coli* host. Among these genes, *bfpA* encodes the bundlin, the major structural subunit of BFP. The bundlin is assembled with the help of typical TFP assembly components, that is, prepilin peptidase (BfpP), traffic ATPases (BfpD and BfpF), secretin (BfpB), PilC-like protein (BfpE), minor pilins (BfpI, BfpJ and BfpK), but also requires the function of components not found in classical TFP systems. Those are a lytic transglycosylase (BfpH) and four proteins of unknown function, BfpG, BfpC, BfpU and BfpL. No homologue of the genes encoding these proteins of unknown function have been found in the genome sequence of *P. aeruginosa* PAO1 or *P. putida* KT2440, revealing that most probably no BFP-type of pili are present in these organisms. The class b of TFP is also defined by the peculiar N-terminal sequence of their major pilin, which is unusually long and lack the mostly conserved phenylalanine at the N-terminus of the mature pilin. The class b of TFP also includes the longus pilus of enterotoxigenic *E. coli* (ETEC)²⁸ and the TCP of *V. cholerae*⁴⁶.

2.7. Archaeal Flagellin

The precursors of archaeal flagellins also contain a leader peptide that is homologous to bacterial type IV pilin signal sequences²⁴. This observation suggested that the assembly and structure of archaeal flagella are similar to those of bacterial pili. Indeed, the core structures of bacterial pili and the flagellum of the halophilic archaeon *Halobacterium salinarum* are similar¹³. Moreover, a prepilin peptidase, called PibD, was recently identified in *Sulfolobus solfataricus*, which is responsible for cleavage of prepilin-like precursors including the *S. solfataricus* flagellin (preFlaB)¹.

3. THE CHAPERONE-USHER PATHWAY

Before coming back more specifically on *P. aeruginosa* chaperone-usher pathways, we will quickly describe the main characteristics of these pathways through data concerning the type P or the type 1 pili assembly. Type P pili are often expressed in *E. coli* associated with acute pyelonephritis, whereas type 1 pili are conserved throughout the *Enterobacteriaceae*. These pili contain adhesins with receptor-binding specificity that contributes to the host and tissue tropism demonstrated by the various bacteria. It is not rare to find a high number of fimbrial assembly pathways in one single organism. The UTI89 (urinary tract infection) *E. coli* isolate possesses up to 12 different gene clusters encoding chaperone-usher pathways.

3.1. The *pap* and *fim* Genes

The *pap* genes (*pap* for pyelonephritis-associated pili) encode components of the P pili, as well as specific regulators controlling their expression³⁷. The *pap* gene cluster encodes a total of 11 proteins among which six structural proteins make up the P pilus. The P pili are composite extracellular structures found all around the bacteria. They consist of a flexible fibrilla joined end to end to a pilus rod. The tip fibrilla is comprised predominantly of PapE subunits arranged in an open helical conformation. The rod is composed of repeating PapA subunits packed into a right-handed helical cylinder, with an external diameter of 6.8 nm, an axial hole of 1.5 nm and a pitch distance of 2.49 nm, with 3.28 subunits per turn of the helical cylinder. The adhesin of the P pili, PapG³⁶, mediates binding to Gal α (1-4)Gal moieties present in the globoseries of glycolipids on uroepithelial cells and erythrocytes. The adhesin is located at the distal end of the tip and is joined to the PapE fibrilla via a specialised adapter protein, PapF. Another adapter protein, PapK, joins the adhesin-containing PapE fibrilla to the PapA rod. Another minor component, PapH, is located at the base of the PapA rod. Its incorporation into the growing structure is thought to anchor the pilus to the cell membrane and to signal the termination of assembly⁴.

The *fim* genes encode components and regulatory elements needed for the assembly of type I pili, which are important virulence determinants expressed in *E. coli* as well as in most members of the *Enterobacteriaceae* family³⁷. They bind specifically to mannose oligosaccharides⁴⁵, and are also involved in attachment to inert surfaces. Like the P pili, they are composite structures in which a short tip fibrillar structure containing FimG and the FimH adhesin is joined via the FimF subunit to a rod comprised predominantly of FimA subunits. The overall structure of the type I rod is very similar to that of the PapA rod of P pili. The type I pilin subunits are arranged in a helix with an external diameter of 6–7 nm and an axial hole of 2–2.5 nm, with a pitch distance of 2.31 nm and 3.125 subunits per turn.

These two extracellular Pap and Fim appendages, assembled by their respective machineries, have common features. The Pap and Fim appendages are thus heteropolymeric structures composed of rigid rods joined to thinner and more flexible tip fibrillae. The formation of the pilus is ordered via a partitioning assembly of the various fimbrial subunits through one unique outer membrane protein, the usher (see Section 3.2). The final position of a fimbrial component in the pilus is being determined in part by the strength with which the pilin-chaperone complex (see Section 3.3) is bound to the usher¹⁹. In this way, the adhesin component is being assembled first. It should be noted that the rod subunit PapA cannot be assembled in the absence of other tip subunits, even though chaperone and usher are present (see Section 3.2).

It is finally worth noting that the regulation of P- and type 1-pili expression is subject to phase variation⁷.

3.2. Molecular Mechanism of Fimbrial Assembly

Two proteins are required for the ordered assembly of the different fimbrial subunits, that is, a periplasmic chaperone (PapD or FimC) and an usher protein localised in the outer membrane (PapC or FimD). Pilin subunits are translocated through the inner membrane via the Sec system²². The periplasmic chaperone then interacts specifically with each of the fimbrial subunits. When the pilin subunit is overproduced, but the chaperone is not co-expressed, subunits are unable to fold and thus aggregate, before being degraded through the action of the DegP protease, whose expression is controlled by the Cpx two-component signal transduction pathway¹⁷. Under the same circumstances, but if fimbrial subunits expression is performed in a *degP* mutant, the subunits remain associated with the inner membrane in an aggregated state. Each chaperone–subunit complex is stable, and this interaction allows the correctly folded subunit to be addressed to the usher protein. This usher protein forms multimeric pores in the outer membrane, with an internal diameter of 2–3 nm⁷². It allows, through the pore, the assembly of pilin subunits in the extracellular pilus-growing structure. The structure could grow outside the bacterium as a linear fibre with a diameter of 2 nm, which is compatible with the pore diameter. The final rod structure, which has a wider diameter of about 6–8 nm, could be formed afterwards by the helical folding of this linear fibre. The name of usher protein comes from its ability to recognise in an ordered manner the different subunits to be assembled. Indeed, the stability of the interaction between the usher protein and the chaperone–subunit complexes varies according to the subunit. In both the P and the type I assembly pathway, the strongest interaction is seen with the chaperone–adhesin complex⁶⁵. This interaction initiates the assembly of the other subunits, probably by inducing a change in the pore conformation. Indeed, it has been shown that the formation of a chaperone–adhesin–usher ternary complex induces a conformational change in the FimD usher to an assembly competent form that is maintained throughout pilus assembly⁶⁵. The usher protein could also be involved in the dissociation of the chaperone–subunit complexes, allowing the subunit incorporation into the growing structure and the recycling of the chaperone toward other subunits.

3.3. Structural Features

3.3.1. The Periplasmic Chaperone. The PapD structure has been determined with a resolution of 2 Å, revealing two immunoglobulin-like domains oriented

in a L-shape³⁵. Each domain is a β -barrel structure formed by two anti-parallel β -pleated sheets. A conserved internal salt bridge is thought to maintain the two domains in the right orientation, giving the protein a boomerang shape. Each domain contains seven β -strands (A1–G1 and A2–G2) arranged in two β -sheets pinned together. Identified chaperones are organised in two subfamilies structurally and functionally distinct³⁸, according to the length of the loop that connects the F1 and G1 β -strands of the N-terminal domain. The two subfamilies are designated FGS (for F1–G1 short) and FGL (for F1–G1 long), corresponding to loop lengths of 10 to 20 amino acids and 21 to 29 amino acids, respectively. Interestingly, these two subfamilies assemble pili with distinct architectures. On the one hand, FGS chaperones, of which PapD and FimC are members, are involved in assembling pili with rod-like architecture (thick and rigid) or fibrilla-like architecture (thin and flexible). FGL chaperones, on the other hand, mediate the assembly of not really well characterised, atypical, or afimbrial, adhesive structures on the surface of bacteria, as the *Yersinia pestis* Caf1 antigen for instance¹¹. It is worth noting that conserved features in the C-terminus of all subunits assembled into rod-like fibres are absent or variable in subunits that are assembled into non-fimbrial or atypical structures.

3.3.2. The Pilin and Adhesin Subunits. Pilin subunits have an incomplete immunoglobulin fold in which the seventh (carboxy terminal) β -strand present in immunoglobulin folds is lacking. The absence of this strand produces a deep groove along the surface of the pilin domain and exposes its hydrophobic core, hence the instability of pilus subunits in the absence of the chaperone. Indeed, in complex with the chaperone, the immunoglobulin fold of the pilus subunit is completed by the G1 β -strand of the chaperone, which is inserted into the groove and protects the hydrophobic core of the subunit. The immunoglobulin fold of periplasmic chaperone are thus utilised by pilus subunits like templates to achieve their correct three-dimensional structures so that they can be assembled into pili.

The FimH adhesin consists of two domains (Figure 3), a receptor-binding domain and a pilin domain¹². The receptor-binding domain, which does not interact significantly with the chaperone, is an elongated 11-stranded β -barrel with a putative mannose-binding pocket at its distal end. The PapG adhesin has been co-crystallised with the membrane receptor isotype GbO4 (globotetraosylceramide)²⁰. The receptor-binding site of PapG has been localised laterally, strongly suggesting a requirement for a tip fibrilla flexible enough to orientate the receptor-binding sites parallel to the membrane. Whereas both adhesins, FimH and PapG, share a similar β -barrel fold in their adhesin domain, they display different ligand-binding regions⁵². The C-terminal pilin domains of FimH and PapG, just like the single domain of the FimA pilin subunit, for instance, have immunoglobulin folds that lack the seventh β -strand and thus contain the chaperone-recognition motif.

3.3.3. The “Donor Strand Complementation” Model. As already mentioned, in the chaperone–subunit complex, the G1 strand of the chaperone and a portion of the F1–G1 loop, which extends to lengthen the G1 strand, complete the immunoglobulin fold of the fimbrial subunit by occupying the hydrophobic groove⁶⁴. This donor strand complementation interaction stabilises the subunit by shielding its hydrophobic core. The immunoglobulin fold that is produced is atypical since the G1 strand of the chaperone runs parallel to the F strand, rather than anti-parallel as it does in a canonical immunoglobulin fold. Mutational and biochemical studies indicate that the groove of the pilin subunit that participates in donor strand complementation with the chaperone is the one that interacts with other subunits once integrated in the pilus structure. Thus, the G1 strand of the chaperone, by occupying the groove, also prevents premature subunits association in the periplasm.

3.3.4. The “Donor Strand Exchange” Model. Pilin subunits have an N-terminal extension with a highly conserved alternating hydrophobic motif that has been shown to participate in subunit–subunit interactions. The N-terminal extension does not contribute to the immunoglobulin fold of the subunit, but rather projects away from the rest of the pilin domain where it would be free to interact with another subunit. Therefore, the N-terminal extension of one subunit may displace the chaperone G1 strand (see Section 3.3.3) from its neighbouring subunit in a mechanism termed donor strand exchange^{12, 64}. The pilin domain of the adhesin lacks this N-terminal extension, consistent with its location at the tip of the pilus. The subsequent assembly order of subunits in the pilus may, at least in part, be determined by the stereo-chemical complementarity between the N-terminal extension of the subunit and the groove of its neighbour. The N-terminal strand would insert anti-parallel to the F strand of the neighbouring subunit, unlike the chaperone G1 strand, which inserts in a parallel fashion. The mature pilus would thus consist of immunoglobulin domains, each of which contributes a strand to the fold of the preceding subunit to produce the pilus fibre. Detailed mechanistic studies revealed that the donor strand exchange reaction requires the presence of the chaperone bound to an incoming subunit, and is necessary for subunit translocation across the outer membrane usher⁵.

4. THE *P. AERUGINOSA* CUP SYSTEMS

4.1. Identification of Chaperone-Usher Pathways in *P. aeruginosa*

The screening of a collection of Tn5 mutants constructed in a non-piliated strain of *P. aeruginosa* allowed the identification of clones which were not able

to form a biofilm on an abiotic surface⁷³. Beside the possible interruption of genes encoding flagellar components, which was shown to be a key process in biofilm formation⁵⁷, some mutants exhibiting a negative adhesive phenotype were interrupted in a cluster of genes encoding a machinery belonging to the chaperone-usher pathway. That cluster of genes was composed of five genes (Figure 2), named *cupA1*–*cupA5*, *cup* for chaperone-usher pathway, among which two genes, *cupA2* and *cupA5*, are encoding periplasmic chaperones identified as members of the FGS family (see Section 3.3.1) and for which the nearest homologues were found to be two chaperones from *E. coli* (F17a-D) and *B. pertussis* (FhaD) with 35% and 34% identity, respectively. It is worth noting that chaperones from the FGS family assemble thick and thin pili, in contrast to chaperones from the FGL family that assemble afimbrial or atypical adhesins at the cell surface (see Section 3.3.1). However, to date no CupA-dependent fimbrial structures have been clearly detected at the bacterial cell surface. The CupA1 typical fimbrial subunit was found to be close to the F17A

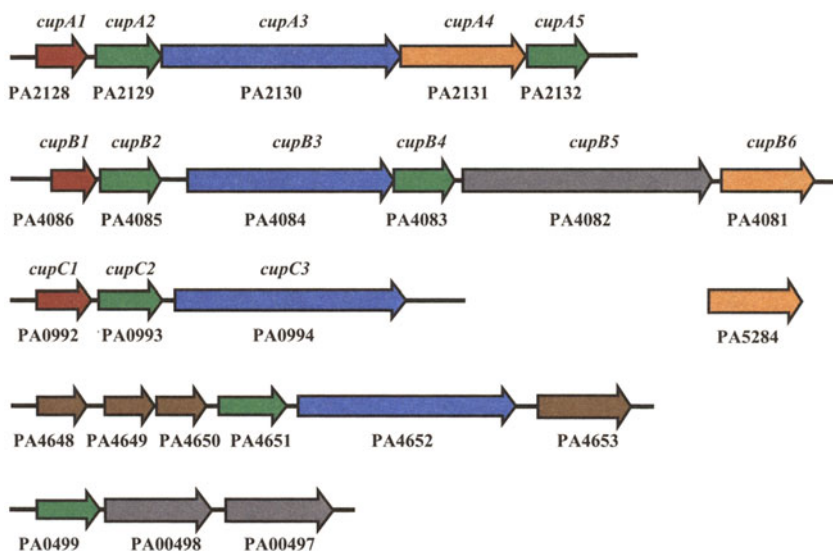


Figure 2. *P. aeruginosa* *cup* and related gene clusters. The genes encoding putative fimbrial subunits are indicated in red, whereas those encoding putative adhesins are indicated in orange. The genes encoding chaperones are indicated in green whereas those genes encoding ushers are in blue. The genes represented in brown are encoding a putative novel kind of fimbrial subunit (COG5430) and those in grey have no similarities with known components of the chaperone-usher pathways. The number corresponding to the gene annotation at <http://www.pseudomonas.com> are indicated below each gene. When appropriate, the gene name has also been indicated above.

fimbrial subunit of *E. coli* sharing 37% identity. The CupA4 protein does not seem to be a highly conserved fimbrial subunit (Figure 3), however it shows some degrees of similarity with putative fimbrial subunits from *Salmonella enterica* and *Salmonella typhimurium*. Moreover, it has a large size (454 amino acids), which suggests that it might be an atypical adhesin protein. Finally, the usher protein is presenting identity with FocD from *E. coli* (41% identity). The role of the cluster of genes encoding the CupA system has been demonstrated in the TFP-deficient strain as well as in the wild-type strain, suggesting that CupA-dependent attachment to abiotic surfaces is independent of the presence of TFP and is essential for initial attachment.

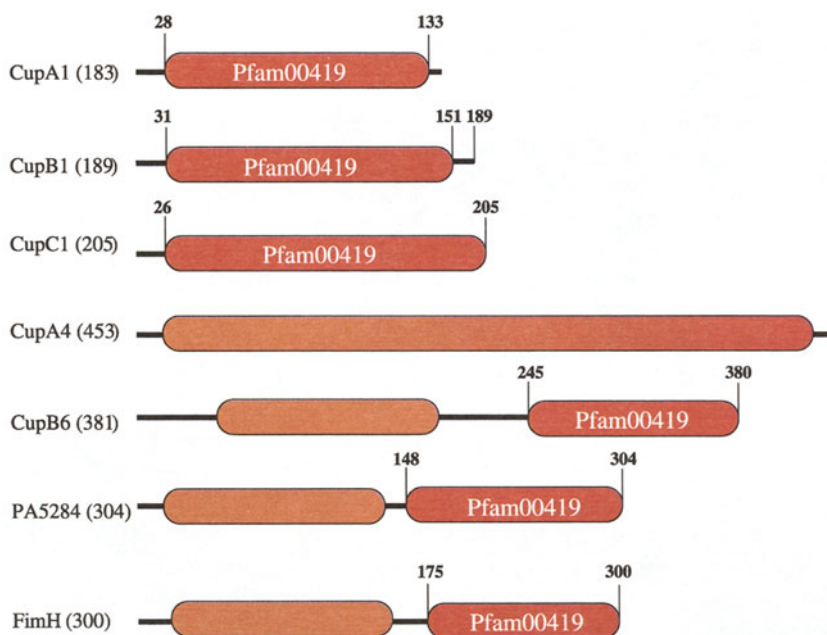


Figure 3. Putative *P. aeruginosa* fimbrial and adhesin subunits. The CupA1, CupB1 and CupC1 proteins contain the canonical fimbrial subunit domain Pfam00419 (also COG3539), which is represented in red. The well-characterised *E. coli* FimH adhesin contains this domain in its C-terminal region, whereas the N-terminal domain contains the carbohydrate-binding site (indicated in orange). CupB6 and PA5284 present the same domain organisation as FimH, even though the representation of the receptor-binding domain is only speculative. In spite of low similarities with fimbrial subunits, no canonical domains have been identified in CupA4. Given the length of the protein, which is rather similar to the size of adhesins subunits, one may speculate that CupA4 is a novel type of adhesin with uncharacterised features. The length of each protein (in amino acids) is indicated between brackets, and the position of the Pfam00419 domain is also indicated.

The analysis of the *P. aeruginosa* PAO1 genome revealed the presence of two additional complete sets of genes encoding components of a chaperone-usher pathway (Figure 2). These two clusters annotated as *cupB* and *cupC* were composed of, respectively, 6 and 3 genes. In the *cupB* cluster, the CupB1 protein is a typical fimbrial subunit homologous to the F17A fimbrial subunit of *E. coli* (37% of identity). The CupB6 protein presents the typical two-domain organisation of a fimbrial adhesin that is exposed at the tip of the fimbriae (Figure 3), like the well-known FimH and PapG adhesins from *E. coli*. The closest similarity is however with the LpfE protein from *S. typhimurium* (25% identity), which is found essentially in the non-adhesin domain of the protein, that is, the C-terminal pilin domain (Figure 3). Further alignment of the putative carbohydrate binding domain of CupB6 protein comprising 230 amino acids with the carbohydrate domains of FimH and PapG revealed an identity not more than 19% with no conserved residue involved in binding to mannose or GbO4. However, the domain organisation of CupB6 strongly suggests that it may be the adhesin of the CupB system (Figure 3). The *cupB5* gene has no similarity with genes located in other *cup* clusters, but shared similarity with proteins that are transported via a two partner secretion system (TPS, see Chapter 26) in particular those which possess adhesive properties such as the filamentous haemagglutinin (FHA) from *Bordetella pertussis*³⁹. The CupB2 and CupB4 proteins are chaperone-like proteins belonging to the FGS family, their closest homologue being the FimB/FhaD precursor from *B. pertussis*. The CupB3 protein is the usher. The *cupC* cluster (Figure 2) contains only three genes encoding the fimbrial subunit CupC1 sharing 28% of identity with type1 fimbrial subunit of *Klebsiella pneumoniae*, a chaperone-like protein CupC2 sharing 37% of identity with EcpD of *E. coli* and the usher CupC3 sharing 34% of identity with the FimD protein from *E. coli*. In contrast to *cupA*, mutations in the *cupB* and *cupC* gene clusters do not alter *P. aeruginosa* biofilm formation on abiotic surfaces⁷³.

Future experiments should aim at determining the specificity in CupA-dependent adhesion, and understanding whether CupB and CupC systems are used by the bacterium for attachment to other surfaces, such as epithelial cells, or for attachment in environmental conditions different as those that have been used in the CupA-dependent attachment assay.

4.2. Regulation of *Cup* Gene Expression

The regulation of the *pap* and *fim* gene clusters is notably controlled by phase variation mechanisms⁷. No regulatory elements as those found in the *pap* cluster, or typical DNA sequences susceptible to be recognised by site-specific recombinases, as for the *fim* gene cluster, have been identified in the upstream region of the *cup* gene clusters, which suggests a different regulatory

mechanism for the expression of these genes. A *cupA-lacZ* promoter fusion revealed that *cupA* transcription was repressed in *P. aeruginosa* upon normal growth conditions. By screening a Tn5 *P. aeruginosa* mutant library, we identified a gene, *mvaT*, whose product apparently represses *cupA* gene expression. MvaT is a novel type of transcriptional regulator, only found so far in *Pseudomonas* species. Global transcriptional profiling using *P. aeruginosa* DNA chips (Affymetrix, Inc.) confirmed that *cupA1* is upregulated by 15-fold in a *mvaT* genetic background (Vallet *et al.*^{73a}). Interestingly, the *cupB* and *cupC* gene clusters appeared to be upregulated by 2–3 fold in the *mvaT* mutant. Since MvaT was previously identified as a repressor of *lecA* expression¹⁸, *lecA* encoding a *P. aeruginosa* lectin, which plays a role in adhesion, it is tempting to suggest that MvaT might be a global regulator of genes related to the bacterial attachment process.

4.3. Other Cup-Related Genes in *P. aeruginosa* and *P. putida* Genomes

Exploring the *P. aeruginosa* PAO1 genome (<http://www.pseudomonas.com>) revealed other clusters containing *cup*-related genes, even though these are incomplete. An incomplete fimbrial assembly system was found, which does not contain a gene encoding the usher but a gene encoding a chaperone (PA0499) (Figure 2). Linked to this gene are two genes (PA0497 and PA0498), which encode signal peptide-containing proteins similar to the secretory protein YbgO from *E. coli*. In the *E. coli* genome sequence, the *ybgO* gene is found clustered with genes encoding chaperone-usher components. Another gene cluster ranging from PA4648 to PA4653 contains a chaperone- and an usher-encoding gene, PA4651 and PA4652, respectively (Figure 2). Intriguingly, no genes encoding typical fimbrial subunits could be identified. However, the four other *orfs* (PA4648, PA4649, PA4650 and PA4653) are encoding proteins that contain a characteristic domain found in the protein U precursor of *M. xanthus*²⁹. This protein is produced as a precursor with a canonical signal peptide and is assembled on the spore surface after crossing the membrane. The protein U domain, called SCPU (pfam05229), is included in a larger conserved domain identified as COG5430. This domain might be representative of a new type of fimbrial subunits assembled by a chaperone-usher pathway. Interestingly, the gene organisation is very similar to the genetic organisation of a gene cluster found in the *P. putida* KT2440 genome, which encompasses genes PP2357-PP2363. In *P. putida* KT2440, the cluster is identified as *csu*-like, a gene cluster also found in *Vibrio parahaemolyticus*, and in which *CsuA*, *CsuB* and *CsuE* may be hypothetical fimbrial subunits. It is worth noting that both *P. aeruginosa* clusters PA0497-PA0499 and PA4648-PA4653 are up-regulated in the *mvaT* background just as the other *cup* gene clusters (Vallet *et al.*^{73a}). Finally, in the *P. putida* KT2440 genome,

another gene cluster encoding a chaperone and an usher is found (PP1890 and PP1889, respectively), which also contains two fimbrial subunits encoding genes, PP1891 and PP1888.

5. THE *P. AERUGINOSA* “TIGHT ADHERENCE” GENE CLUSTER

5.1. Genomic Considerations

P. aeruginosa genome sequence analysis revealed additional clusters of genes encoding putative fimbriae assembly machineries. Among these, we found a gene cluster (PA4297–PA4306) encoding a Flp protein (Fimbrial low molecular-weight pili subunit) and components of a so-called Tad assembly machinery. This gene cluster is highly identical to the *tad* cluster of *A. actinomycetemcomitans*, a gram-negative bacterium responsible for localized juvenile periodontitis⁴⁰. The *tad* genes in this organism are involved in tight and non-specific adherence mediated by Flp pili, which results in the formation of extremely tenacious biofilms on a variety of solid surfaces and more particularly the teeth. The Flp pili are long, thick fibrils composed of bundles of thin pili⁴¹. In *A. actinomycetemcomitans*, the *tad* locus regroups 14 genes in a 12 kb region flanked by the *flp-1* gene and the *tadG* gene⁶¹. The *flp-1* gene encodes a protein of 7.5 kDa, the final mature size being 6.5 kDa, which localizes to *A. actinomycetemcomitans* fibrils. In addition to *flp-1*, another gene, *flp-2* encodes a fimbrial subunit, with 52% identity to Flp-1, but presence of Flp-2 in the fibrils has not been demonstrated. In *P. aeruginosa* only one *flp*-like gene is found (PA4306) which is transcribed in the reverse orientation as compared to the other *tad* genes. The Flp proteins belong to the class b of type IV pilin subunits (see Section 2.6), but based on phylogenetic evidences, they may form a distinct subfamily. The members of this family are characterised by a relatively small size (smaller than 90 amino acids long), a shorter carboxy-terminal domain, as compared to other type IV pilins, and an invariant tyrosine residue immediately following the conserved glutamate at the +5 position. The *A. actinomycetemcomitans* and the *P. aeruginosa* *tad* loci contain genes encoding highly conserved proteins required for TFP assembly. These components are a traffic ATPase (PA4302/HvbA or TadA), a secretin (PA4304/XqhC or RcpA) and two PilC-like proteins (PA4300 and PA4301 or TadB and TadC). The name HvbA results from a previous annotation (<http://www.pseudomonas.com>) indicating that PA4302 encodes a traffic ATPase highly homologous to *VirB11* from *Agrobacterium tumefaciens*⁶², whereas XqhC indicated that PA4304 encodes a secretin which is an *XcpQ* homologue. It is worth noting that among those systems requiring both a

traffic ATPase and a secretin that is the first report for the requirement of two-independent PilC-like proteins, which may suggest that this family of polytopic inner membrane protein may function as homo- or heteromultimers within the assembled machinery. Like for the class b of TFP another set of components is required that is not conserved in all type IV-like pili assembly systems. These components are encoded by the genes annotated PA4305 (putative *rcpC*), PA4299 (putative *tadD*), PA4298 and PA4297 (putative *tadG*). Finally, it is not known whether the *P. aeruginosa* prepilin peptidase used for maturation of Flp pili is the one used for classical TFP (PilD/XcpA) or whether there exists a specific peptidase for these Flp prepilins. In the case of the *A. actinomycetemcomitans* *tad* gene cluster, the *tadV* gene is encoding a putative prepilin peptidase whose homolog in *Caulobacter crescentus* (*cpaA*) has been shown to be required for pilus production⁶⁸. In the *P. aeruginosa* genome, the gene annotated PA4295, located immediately downstream the *tad* gene cluster, but transcribed in the opposite direction, encodes a protein with similarities to the *cpaA* and *tadV* gene products and may be involved in Flp prepilin maturation.

5.2. Role of the *Tad* Gene Cluster in *P. aeruginosa*

The *P. aeruginosa* *flp* gene has been cloned and overexpressed in the PAO1 strain or a non-adherent strain PAK::*fliCpilA*, devoid of TFP and flagella. Using a simple test for biofilm formation, we observed that *flp* overexpression in the PAO1 strain, enhanced the adherence ability of about 25%, while the non-adherent PAK::*fliCpilA* strain clearly became adherent in these conditions. Furthermore, we could observe some bundled pili in the *flp*-overexpressing PAO1 strain by transmission electron microscopy using classical negative staining. Finally, mutant in the *flp* gene had a delay in biofilm formation compared to the wild-type strain. These results support the functionality of Flp pili in *P. aeruginosa* biofilm formation (Marianne Aurouze, unpublished).

6. GENERAL CONCLUSIONS

One of the earliest events, which occur in the course of a bacterial infection is the molecular interaction that takes place between the pathogen and the host. The colonisation of host tissues is mediated by adhesins located at the surface of the bacterium, which then recognize and bind to specific receptors moieties present on the host cells. In many cases, these adhesins are assembled into hair-like appendages called pili or fimbriae, which append at the bacterial cell surface. Few mechanisms have been characterised that account for the

assembly of these complex structures. Among these, the chaperone-usher pathway and TFP biogenesis have been extensively described in this chapter.

The chaperone-usher pathways (Cup) identified in *P. aeruginosa* are slightly different from the P pili (Pap) or type 1 pili (Fim) assembly pathway since no minor pilins have been identified. Indeed, in the *cupA* and *cupB* clusters one gene is encoding a putative major fimbrial subunit whereas another gene encodes a putative adhesin. Each subunit, the major pilin and the adhesin, might have their dedicated chaperone, whereas all the Pap and Fim fimbrial subunits utilize one single chaperone molecule. It is thus questionable whether tip fibrillae, such as those found in Pap and Fim systems, are assembled by the Cup pathway, and whether adaptors pilins are requested to link the adhesin to the putative pilus-rod structure. However, the Cup chaperones belong to the FGS family, which suggests a role in the assembly of fibrillar structure. In the Pap and Fim systems, the major subunit forms the rigid rod structure, which allows the adhesin to be extruded from the lipopolysaccharide (LPS) and exopolysaccharide layer that surround the bacterium, whereas the flexible fibrilla allows the positioning of the adhesin with respect to the host cell. The Cup systems may not use such finely tuned strategy to expose their putative adhesins at the bacterial surface. Moreover, in the case of the CupC cluster, only one major fimbrial subunit-encoding gene was found. This does not exclude that a gene-encoding adhesin, which may be part of the CupC system, would be found at another chromosomal locus. In this respect, the gene annotated PA5284 is encoding a fimbrial subunit with the characteristic two-domains organisation of the fimbrial adhesin, which consists of a specific N-terminal domain that precedes the highly conserved C-terminal pilin domain (Figure 3).

The TFP were until recently presented as the main hair-like appendages of *P. aeruginosa*. These pili are assembled via a mechanism which is highly related to other systems involved in the transport of macromolecules and more particularly the type II protein secretion system (see Chapter 26). These systems use a traffic ATPase, a secretin, a canonical polytopic inner membrane protein, one, or more, pilin or pilin-like protein, and a prepilin peptidase. Interestingly, it was shown that in the type II protein secretion system, overproduction of the major pilin-like protein, XcpT, results in the assembly of multifibrillar appendages at the cell surface, which confer increased adhesiveness to the bacterium and whose function in term of protein secretion is still to be elucidated²³. However, both TFP assembly and type II protein secretion processes require additional, distinct and specific components. This is also true for the assembly of class b of TFP, such as BFP, TCP, or the subfamily of Flp pili. It is worth noting that the correlation between TFP and type II protein secretion might be extended, since it was shown recently that secretion of a soluble colonization factor uses the TCP biogenesis pathway in *V. cholerae*⁴⁴.

Other pathways are known⁶⁹, such as the extracellular nucleation-precipitation pathway³¹. Such pathway is used for the assembly of so-called curli. Curli are thin aggregative fimbriae found notably in *E. coli* and *Salmonella*. Formation occurs from the outside by precipitation of secreted pilin subunits (CsgA) into thin fibres. Precipitation requires a nucleator protein (CsgB) that can also be produced by adjacent cells. Additional assembly components, CsgE, CsgF and CsgG are required. No curli genes have been found in the *P. aeruginosa* PAO1 genome. However, analysis of the *P. putida* KT2440 genome sequence revealed the presence of two clusters, one encoding CsgA and CsgB homologues, PP3399 and PP3398, respectively, whereas homologues of the CsgE, CsgF and CsgG components are encoded in another gene cluster, PP3474, PP3473 and PP3472, respectively. Interestingly these three genes seem to be located in the same cluster that also contains genes encoding TFP-like proteins (see Section 2.5). Another pathway, involved in assembly of CS1 pili by *E. coli*, has been described²⁶. In this case, the pathway employs specialized periplasmic chaperones (CooB), which binds the major pilin subunit (CooA) and the tip subunit (CooD). CooC is an outer membrane protein that may serve as a channel, but CooB and CooD do not share the characteristic features of the chaperone-usheer components that we described before. For this reason, the Coo-dependent pathway is also called the alternate chaperone pathway. However, it was shown that CooA is efficiently transported to the extracellular medium in the absence of CooBCD, which suggests a possible nucleation mechanism during the assembly process. No genes encoding Coo homologues could be found in the *P. aeruginosa* PAO1 and *P. putida* KT2440 genome sequences. Finally, the formation of cable pili assembled by *Burkholderia cepacia* strains isolated from cystic fibrosis patients are dependent on the *cbl* genes⁶³, which are related to the genes required for CS1 pili assembly in *E. coli*. The name cable pili is due to a novel morphological appearance of these pili as giant intertwined fibres.

In conclusion, the arsenal of putative fimbrial adhesins that have been found in *P. aeruginosa* might reflect its ubiquity. These complex structures may be required for attachment on different surfaces, at different stages of infection or in different environmental conditions. The way these different fimbrial structures are assembled need to be extensively studied and may be used in the future for therapeutics possibilities since pilus biogenesis is closely linked to host pathogenesis.

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PROTEIN SECRETION MECHANISMS IN *PSEUDOMONAS*

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1. GENERAL PRINCIPLES FOR PROTEIN SECRETION IN GRAM-NEGATIVE BACTERIA

The cell envelope of Gram-negative bacteria is composed of two membranes, which are separated by the peptidoglycan-containing periplasm. Whereas the envelope forms an essential barrier against harmful substances, such as drugs, from the external medium, it is nevertheless a compartment of intense molecular traffic. Such traffic is for example required to allow for the acquisition of essential nutriment from the external medium or to allow for the extrusion of toxic substances. Essential enzymatic reactions that have to take place in the external environment and that should be catalysed by the bacteria themselves require the delivery into the medium of enzymes initially synthesized in the cytoplasm. These enzymes have to cross the cell envelope to reach their destination, and for this dedicated process, we will use the term “protein secretion.”

Numerous studies dealing with the molecular mechanism of protein secretion have revealed that Gram-negative bacteria evolved different strategies to achieve this process. These mechanisms have been classified with respect to a number of characteristics. Above all, two main strategies may be distinguished. In some cases, a periplasmic intermediate can be identified, subsequent to inner membrane translocation and preceding translocation across the outer membrane. These so-called two-step mechanisms include type II systems⁵⁴, autotransporters⁷⁵ and two-partner systems⁸⁴. In contrast, the type I⁶² and type III²⁶ secretion systems are bypassing the periplasm and are thus called one-step processes. The status of the type IV system has not clearly been established, since some type IV substrates, like the pertussis toxin, transit through the periplasm to the extracellular medium, whereas the *Helicobacter pylori* CagA is, without a periplasmic intermediate, directly injected from the bacterial cytoplasm into the host cell²². Since there is no type IV secretion system identified in *Pseudomonas aeruginosa*, it will not be described in this chapter, and we will focus on the five other systems that are all present in *P. aeruginosa*.

2. TYPE II SECRETION SYSTEM

2.1. General Features and Nomenclature

The type II secretion mechanism is part of a two-step process. Exoproteins following this pathway are synthesized as signal peptide-containing precursors. The precursors are targeted to the Sec apparatus and translocated across the cytoplasmic membrane¹¹⁹. After cleavage of the signal peptide, the mature exoproteins are released into the periplasm, where they fold with the help of general or dedicated chaperones and foldases. The type II machinery, also known as the “Secreton,” is responsible for the translocation of the periplasmic intermediates across the outer membrane into the extracellular milieu. At this stage, it is not obvious that the exoprotein is freely released into the periplasm before it travels through the type II apparatus. An alternative possibility is that it is already engaged with the secreton during translocation through the inner membrane.

The Sec pathway for translocation across the cytoplasmic membrane was named the general export pathway (GEP)¹³⁶. As a consequence, the type II secretion system, which extends the transport of the Sec-dependent proteins through the outer membrane, was named (the main terminal branch of) the general secretory pathway, or GSP¹³⁶. It should be mentioned that it is now demonstrated that the type II apparatus can also be supplied with substrates by another inner membrane translocation apparatus, known as the Tat system¹⁶².

This Tat system is dedicated to the transport of folded, co-factor containing proteins with a special signal sequence featuring a twin-arginine motif¹²⁶. Hence, the designation “general secretory pathway” is not entirely accurate.

The type II system is broadly conserved in Gram-negative bacteria. It was first discovered in *Klebsiella oxytoca* for pullulanase secretion⁴³. Later on, it was found in *P. aeruginosa*, in which it appears to be used for the secretion of a wide range of toxins and enzymes⁵⁴. In *K. oxytoca*, the secretion components are called Pul, whereas they are called Xcp in the case of *P. aeruginosa*. Often, the general term Gsp is used. The fourth letter of the designations of the homologous components of the different systems is usually identical, for example, PulE of *K. oxytoca* is homologous to GspE of *Escherichia coli*. Unfortunately however, for historical reasons, the fourth letter of the designations of the Xcp components is deviating, for example, XcpR is homologous to PulE. Since the *P. aeruginosa* secretion is one of the most studied systems, it will be used for the general description of the type II pathway. For clarity, we will slightly modify the nomenclature, for example, we will use XcpR_E indicating that XcpR belongs to the GspE family.

2.2. The Components of the Xcp Secretion Apparatus

The functioning of the Xcp system of *P. aeruginosa* involves at least 12 different proteins⁵⁴. The genes encoding these proteins are located on two distinct chromosomal loci. One of these loci contains 11 genes, named *xcpP_C* to *xcpZ_M*, whereas the second locus contains one single *xcp* gene named *xcpA_O*. Subcellular localization, bioinformatics and topological studies revealed that 11 out of the 12 *xcp*-gene products are integral or peripherally associated inner membrane proteins.

2.2.1. The Prepilin Peptidase XcpA_O and the XcpT_GU_HV_IW_JX_K Pseudopilins. There is a strong resemblance between type II secretion and the biogenesis of type IV pili¹¹². The maturation of the pilin subunit PilA requires the function of the prepilin peptidase PilD, which cleaves off a small, 6 amino-acid residue long leader peptide from the pilin precursor¹⁰⁷. It was found that the gene encoding PilD, which is located in a cluster containing other genes involved in type IV pili biogenesis, that is, *pilA*, *pilB*, and *pilC*, is identical to that also identified as XcpA_O. Thus, mutations in the *xcpA_O/pilD* gene hampered both type IV piliation and type II protein secretion. XcpA_O is a polytopic inner membrane protein. The role of XcpA_O in type II secretion was shown to be associated with the maturation of five pilin-like proteins, the XcpT_GU_HV_IW_JX_K proteins, which are called pseudopilins^{6, 13, 124} because of their resemblance to PilA. These proteins have the canonical N-terminal leader peptides of type IV pilins, which are 6–7 amino acids long with a global positive charge. The cleavage

site is located right after an invariant glycine residue and precedes a mostly conserved phenylalanine residue. The long hydrophobic stretch found downstream of the cleavage site always contains a glutamate at the +5 position, except in the atypical XcpX_K pseudopilin family. Localization experiments have shown that the pseudopilins are associated with the inner membrane, probably anchored *via* their hydrophobic N-terminal segment. However, in some cases, the mature pseudopilins were also found in the outer membrane fraction⁶. Finally, pseudopilins were shown to form oligomers, thus sharing this property with type IV pilins.

2.2.2. The Traffic ATPase XcpR_E. The XcpR_E protein is a member of the traffic-ATPase family. This family of proteins, which also includes PilB, required for type IV pili biogenesis, is involved in the transport of macromolecules (proteins and DNA) or macromolecular complexes (oligomers, pili, phages, nucleoprotein complex) through the cell envelope of Gram-negative and -positive bacteria¹³² (Table 1). The protein does not contain any hydrophobic segment or signal peptide, which indicates that it stays in the cytoplasm. XcpR_E contains a putative ATP-binding site with a well-conserved Walker A motif. The Walker B motif is poorly conserved, and, as was shown with the Pule homologue, mutations within this region affect the function of the protein only marginally¹³⁴. However, the hallmark of the GspE family resides in the presence of aspartate boxes located between the Walker A and B motifs, which are essential for the function. Even though mutations in the Walker A motif abrogate XcpR_E function, it has proved to be extremely difficult to evidence ATPase activity of the protein. In the case of the GspE members devoted to the type II secretion pathway, biochemical evidence for an autokinase activity has only been obtained for EpsE from *Vibrio cholerae*¹³⁸. However, ATPase activity has clearly been demonstrated for traffic ATPases involved in the type IV secretion pathway, such as *H. pylori* HP205, which is required for CagA secretion, or VirB11, which is involved in T-DNA transfer by *Agrobacterium tumefaciens*^{23, 140}.

Table 1. Appearance of traffic ATPases and secretins in various transport systems.

System	Traffic ATPase	Secretin
Type II secretion	+	+
Type III secretion	—	+
Type IV secretion	+	—
Type IV pili	+	+
Filamentous phage	—	+
DNA uptake Gram [—]	+	+
DNA uptake Gram ⁺	+	—

2.2.3. The Secretin XcpQ_D. The XcpQ_D protein is a member of the secretin family, which also includes PilQ, required for type IV pilin biogenesis⁹. Like the traffic ATPases, secretins are involved in the transport of macromolecules and macromolecular complexes (Table 1). XcpQ_D is synthesized as a signal peptide-containing precursor, and the mature form is assembled as a dodecameric complex in the outer membrane^{10, 17}. Electron microscopic evaluation revealed that the oligomer has a ring-like structure with a central cavity of about 9.5 nm, which may represent the protein-conducting channel. In some cases, but not for XcpQ_D, the assembly of the secretin into the outer membrane requires the function of a chaperone, designated "pilotin," which is a small lipoprotein. This was best described for the PulS chaperone, which protects the PulD secretin from proteolytic degradation and favours its outer membrane insertion⁷². The chaperone-binding domain is located at the C-terminal end of the secretin. It should be noted that the PulD oligomeric complex in the outer membrane still contains the PulS chaperone in a 1 : 1 stoichiometry¹²². The presence of PulS in the final functional secretin implicates that the functional designation of the pilotins as chaperones is not entirely correct.

2.2.4. The XcpS_F Protein. XcpS_F is a polytopic inner membrane protein with three predicted membrane-spanning segments. Interestingly, also XcpS_F has a homologue in the Pil system, which is PilC, whose function has not yet been elucidated.

2.2.5. The Bitopic Inner Membrane Proteins XcpP_C, Y_L and Z_M. Three Xcp proteins have no obvious homologues involved in type IV pili biogenesis. One could imagine that these components might have functions specific for type II secretion. However, XcpY_L, which is anchored into the inner membrane protein *via* its single hydrophobic domain, is required for the peripheral association of the traffic ATPase XcpR_E to the membrane⁴. Moreover, it was shown that another bitopic inner membrane protein, XcpZ_M, is required for the stabilization of the XcpY_L protein¹¹⁶. The stabilization between XcpZ_M and XcpY_L appeared to be mutual. XcpP_C is the least conserved component, when the type II secretion systems of various Gram-negative bacteria are compared. The percentage of identity observed ranges from 13%, when XcpP_C is compared with *K. oxytoca* PulC, to 34%, when it is compared with *Pseudomonas alcaligenes* XcpP_C. XcpP_C is a bitopic inner membrane protein, whose C terminus largely extends into the periplasm. It has been shown that XcpP_C is interacting on the one hand with the XcpQ_D secretin located in the outer membrane and on the other hand with the inner membrane components XcpY_L and XcpZ_M^{12, 63}. Thus, XcpP_C may bridge the inner and outer membranes within the secretin.

2.3. Molecular Mechanisms

According to the description and the localization of the different Xcp proteins, a model is proposed to describe the sequence of events during secretion assembly and functioning.

2.3.1. Assembly of the Secreton. The first event in the assembly of the secreton is to determine its location within the cell envelope. It has been shown in *V. cholerae* that the Eps secreton, just like the type IV pili in *P. aeruginosa*, is polarly located. The Gsp component that defines this location is the XcpZ_M homologue EpsM¹⁴⁴. Even though the location of the *P. aeruginosa* secreton has not yet been reported, it is plausible that XcpZ_M will initially insert into the membrane and nucleate the Xcp secreton at the poles. XcpZ_M will be stabilized in the membrane through its interaction with XcpY_L, which in turn will dock the traffic ATPase XcpR_E at the periphery of the cytoplasmic membrane.

2.3.2. Pseudopilus Assembly. The XcpT_GU_HV_IW_JX_K pseudopilins may be translocated across the cytoplasmic membrane via the Sec system, and the pool of membrane-embedded pseudopilins may subsequently be assembled into a pilus-like structure by the action of the traffic ATPase XcpR_E, probably in concert with XcpS_F. Alternatively, XcpR_E and XcpS_F may both translocate the pseudopilins and assemble the pilus, without a role for the Sec system. Both ideas fit with the role of the traffic ATPase PilB and the integral membrane protein PilC in the assembly of type IV pili and with the fact that genes encoding traffic ATPases are usually tandemly associated with a gene encoding a member of the GspF family. Moreover, it has been shown that XcpR_E is interacting with the XcpT_G pseudopilin⁸⁹. With respect to the stoichiometry, XcpT_G is the most abundant pseudopilin¹²⁴. It has been demonstrated that XcpT_G, when overproduced, is able to form a polymeric structure resembling a pilus⁴⁹. The structures of several traffic ATPases, such as HP205, which is involved in type IV secretion in *H. pylori*, have been solved¹⁷⁶. These structures revealed hexameric rings with a central channel. It has been proposed that, upon ATP hydrolysis, the structure may make a circular move, just like the diaphragm of a camera¹⁴⁰. If XcpR_E has the same structure, it could be suggested that it will help pushing the pseudopilin subunits through a cavity formed by the traffic ATPase complex and, possibly, also XcpS_F, resulting in membrane translocation and pseudopilus formation. The length of the pseudopilus may be controlled, and it is most probably not extruding out of the bacterial cell under physiological conditions. Such length control might be the result of the incorporation of the minor pseudopilins¹⁶¹.

2.3.3. Secretin and Control of the Secretion Gate. The cavity within the ring-like structures formed by the secretins undoubtedly constitutes the

ultimate channel in the outer membrane for giving the exoprotein access to the extracellular medium. Regarding the width of the channel (95 Å), preservation of the barrier function of the outer membrane will require a tight control of channel opening. Assuming that the pseudopilus extends into the periplasm and engages with the channel, it might be one part of such control mechanism. Moreover, since type IV pili are retractile¹⁴⁵, it has even been suggested that assembly/disassembly cycles of the pseudopilus might help pushing exoproteins through the secretin channel⁵⁴. However, since *xcpT_G* mutants are not leaky, additional control of the secretin channel is expected. Additional control has been attributed to XcpP_C, which interacts with XcpQ_D via its C terminus^{12,63}. Such interaction possibly leads to conformational changes within the secretin complex that modulate the size of the channel. Conformational changes in outer membrane receptors have been proposed, for example, in the case of the TonB-dependent uptake of iron–siderophore complexes in *E. coli*¹³⁵. TonB, like XcpP_C, is a bitopic membrane protein interacting via its N-terminal membrane anchor with two other integral membrane proteins, ExbB and ExbD, and through its periplasmic C-terminal domain with a siderophore receptor located in the outer membrane. It couples the energy of the proton gradient across the inner membrane to the transport of the siderophore across the outer membrane. Since XcpP_C also interacts with Xcp components in the inner membrane, conformational changes in the secretin might be initiated at this level to modulate channel opening.

2.4. Secretion Signal

The targeting of substrates to the transport machinery is a prerequisite for all transport mechanisms. Such targeting can be more or less specific. Although the type II secretory apparatus of *P. aeruginosa* seems to be very versatile, since it is able to transport very different proteins, such as elastase, exotoxin A, lipase, phospholipases and a chitin-binding protein, there is also a high level of specificity, since secretion of an exoprotein expressed in a heterologous production host is a rare event. However, this host specificity is usually much less stringent, when closely related organisms, such as *P. aeruginosa* and *P. alcaligenes*³⁹ are investigated. Up to now, no one in the field could identify any consensus sequence or a well-defined domain that alone might be considered as a secretion motif. Exotoxin A consists of three domains, domain I required for host cell binding, domain II for host cell translocation, and domain III harbouring the ADP-ribosyl transferase activity. Several studies aimed at identifying the secretion motif within this protein, divergently pointed to the importance of either one of these three domains^{108, 115, 164}. Thus, probably, folding brings distal regions of the protein together, thereby establishing a structural secretion motif and/or a secretion-competent conformation.

This proposal is in agreement with the observation that periplasmic folding of exoproteins is a prerequisite for their secretion¹⁶, and with the large size of the secretin channel that may accommodate folded polypeptides. Periplasmic folding requires the function of general folding catalysts, such as the disulphide bond isomerases¹⁵. Additionally, dedicated chaperones or foldases may be required. Examples of such dedicated chaperones in *P. aeruginosa* are the propeptide of elastase¹⁶, the Lipase-specific foldase (Lif)⁵⁰, and, possibly, the PlcR protein for the haemolytic phospholipase²⁸. The 3D structures of several type II-dependently secreted proteins are now available. No reliable program allows for the visualization of clear conserved structural motifs between these proteins. One suggestion is that these proteins have a high β -sheet content¹³⁷. However, a number of type II-dependent proteins, such as the *P. aeruginosa* lipase, have only a moderate β -sheet content, whereas some resident periplasmic proteins, such as LolA¹⁵⁰, mostly contain β -sheets. Since the nature of a secretion motif appears extremely difficult to identify, it may be considered that some physico-chemical properties of the exoproteins, such as overall surface charge, might be crucial for type II-dependent secretion.

The notion of substrate specificity also implies that the exoproteins are docked onto a dedicated component of the Xcp apparatus. The only two Xcp components that are not exchangeable, even not between closely related species, are XcpP_C and XcpQ_D^{39, 104}. Accordingly, these are the two best candidates for exoprotein recognition.

2.5. Genomic Considerations

The release of the *P. aeruginosa* PAO1 genome¹⁴⁷ has been greatly supported by a series of annotations referring to various classes of proteins and molecular processes (<http://www.pseudomonas.com>). One of the approaches used to annotate these secretion genes was to BLAST the conserved XcpR_E and XcpQ_D proteins against the whole PAO1 genome. The results pointed to eight genes putatively encoding traffic ATPases and seven genes putatively encoding secretins (Figure 1). Four of the eight genes for traffic ATPases were previously characterized, that is, those encoding XcpR_E, PilB, PilU and PilT (the latter being involved in type IV pili retraction), whereas the other four, *hxcR*, *hvbA*, *hplR* and *hxrA*, were unknown. Similarly, four of the secretin genes were previously characterized, *xcpQ_D*, *pilQ*, *pscC* (involved in type III secretion; see Section 6) and *xqhA* (involved in type II secretion; see Section 2.5.1), whereas three of them were unknown.

2.5.1. The Secretin *XqhA*. Each *xcp* gene has been shown to be essential for the Xcp-mediated type II secretion. Inactivation of any of the 12 genes abolishes secretion of elastase, exotoxin A or lipase, for example. However, David

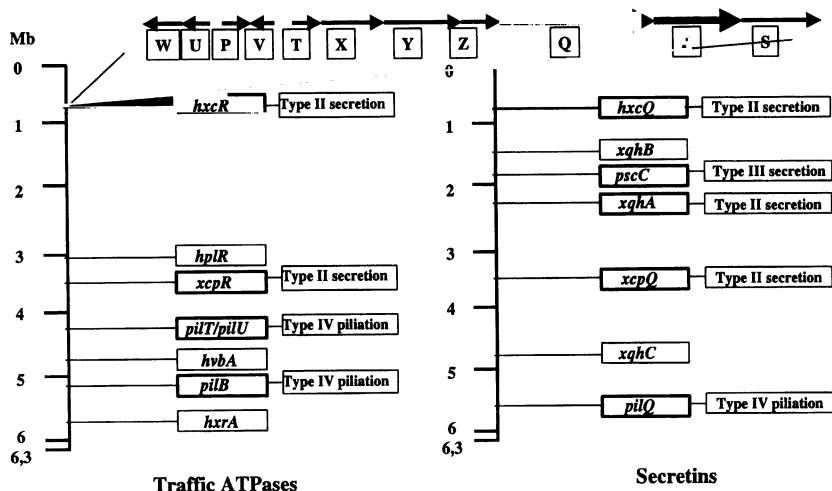


Figure 1. Position of traffic ATPase- and secretin-encoding genes on the PAO1 genome. Genes whose function has been demonstrated are in thick boxes. The entire *hxc* gene cluster has been enlarged and shows all *xcp* gene homologues.

Nunn and co-workers established growth conditions under which Xcp-dependent secretion is essential for bacterial cell viability¹¹⁰. The assay is based on the utilization of olive oil as the sole carbon source. Bacteria that do not secrete lipase are unable to grow on this medium. This sensitive assay revealed that the XcpQ_D function was not absolutely essential for growth. However, an *xqhA xcpQ_D* double mutant was unable to grow on this medium. Apparently, the *xqhA* gene (*XcpQ* homologue A) encodes a secretin that, under certain conditions and for certain substrates, can substitute for the XcpQ_D function and thus be part of a functional Xcp system.

Previous studies revealed that XcpP_C and XcpQ_D could not be exchanged individually between *P. alcaligenes* and *P. aeruginosa*³⁹. However, when introduced simultaneously, the XcpP_C and XcpQ_D proteins from *P. alcaligenes* could functionally be integrated into the *P. aeruginosa* Xcp complex. Thus, XcpP_C and XcpQ_D may function in a coordinate manner by mutual interactions. Interestingly, the gene next to *xqhA* putatively encodes a protein with similarity to XcpP_C. This protein was named XphA (*XcpP* homologue *A*). Although no data are available showing that the gene is actually transcribed, the protein most likely functions in concert with XqhA in type II secretion.

2.5.2. The *Hxc* Type II Secretion System. The *hxcR* and *hxcQ* genes are located in each other's vicinity on the chromosome. Further analysis of the region revealed the presence of nine additional *hxc* genes with striking homologies to

the *xcp* genes⁵, hence the name of the genes (homologous to *Xcp*). However, the genetic organization of the cluster is entirely different. Whereas the *xcp* cluster consists of two oppositely oriented operons, *xcpPQ* and *xcpRSTUVWXYZ*⁵⁴, the order of the genes appears to be completely scrambled in the *hxc* cluster (Figure 1). It was obviously expected that the Hxc system would be involved in type II protein secretion. Indeed, the Hxc system appeared to be required for the secretion of a so-called low-molecular weight alkaline phosphatase¹⁵², which was renamed LapA. The *lapA* gene is located immediately downstream of the *hxc* gene cluster. Interestingly, the gene, immediately downstream of *lapA*, designated *lapB*, is encoding a very similar alkaline phosphatase, and, therefore, LapB as well might be Hxc substrate. Thus, whereas the Xcp system is used by a wide variety of toxins and enzymes, the Hxc system seems to be dedicated to the secretion of low-molecular weight alkaline phosphatases. This suggestion is in agreement with the observation that the *hxc* genes are expressed under low-phosphate conditions. It has been demonstrated that the XcpT_G pseudopilin homologue, HxcT, is processed by the XcpA_O/PilD prepilin peptidase⁵. Thus, this enzyme is required for the functionality of at least three different pathways, Pil, Xcp and Hxc. Consistently, genome analysis did not reveal any other putative prepilin peptidase-encoding gene.

2.5.3. Other Systems. All other secretins and traffic ATPases identified via the PAO1 genome analysis are unlikely to be part of functional type II secretion systems. Possibly, they represent rudimentary gene clusters, which have lost several genes and, thereby, functionality during evolution. Such a rudimentary type II secretion cluster was also described on the *E. coli* K-12 chromosome¹⁵³. One of these gene clusters contains six genes, putatively encoding a traffic ATPase (HplR), a GspF member (HplS), and five pseudopilins HplT, U, V, W and X. However, this cluster lacks genes encoding the type II-specific GspC, GspL and GspM homologs. Moreover, no gene encoding a secretin is directly associated to this cluster. Another locus contains a gene encoding a traffic ATPase (*hvbA*) associated with a secretin gene (*xqhC*). In this case, the two genes belong to a large cluster of genes homologous to *tad* genes of *Actinobacillus actinomycetemcomitans* involved in so-called bacterial tight adherence⁸⁸. The genes putatively encoding the HxrA traffic ATPase and the XqhB secretin are not linked to other putative secretory proteins.

2.6. Type II Secretion in *Pseudomonas putida* and Other Pseudomonads

DNA hybridization experiments revealed the presence of *xcp* homologues also in non-pathogenic rhizosphere Pseudomonads, such as *P. putida*

and *Pseudomonas fluorescens*³⁷. The *xcp* genes of the plant growth-stimulating *P. putida* strain WCS358 were characterized^{38, 40, 41}. Like in *P. aeruginosa*, the *xcpA* gene, encoding the prepilin peptidase, is located in a cluster of pilin biogenesis genes³⁸.

The main cluster of *xcp* genes is differently organized than in *P. aeruginosa*, where the cluster contains two divergently transcribed operons, *xcpPQ* and *xcpRSTUVWXYZ*. The *xcp* cluster in *P. putida* WCS358 contains a large operon encompassing the genes *uxpA-xcpPQRSTUVWXYZ-gspN*^{40, 41}. An *xcpX* homologue was not detected in this operon, whereas, on the other hand, a homologue of the *gspN* gene, which is present in the type II secretion-gene clusters of many bacteria, but not in the *P. aeruginosa xcp* cluster, was present. The product of the first gene of the operon, *uxpA* (upstream of *xcpP*), supposedly is the substrate for the Xcp system of *P. putida*. BLAST searches revealed this protein to be a homologue of the SoxB sulphur oxidation proteins produced by many bacteria. Upstream of *uxpA*, in the opposite orientation, a partial open reading frame, designated *uxpB*, was found⁴⁰. The encoded protein shows high similarity to a predicted phosphatase of *Vibrio vulnificus* (Accession number AA007751). Inspection of the completed genome sequence of *P. putida* strain KT2440¹²¹ revealed a similar organization of the *xcp* cluster in this organism. Interestingly, the *xcpX* gene, which was missing in the analysed *xcp* cluster of strain WCS358, was found downstream of *uxpB* (PP1042). Considering this genetic organization, it seems likely that also UxpB, like UxpA, is a substrate for the *P. putida* Xcp system. Interestingly, both UxpA and UxpB are produced with a signal sequence ending with a lipobox, suggesting that they are lipidated after transport across the inner membrane. Moreover, both signal sequences contain a twin-arginine motif, suggesting that they are transported across the inner membrane via the Tat system, rather than via the Sec system. This supposition is reinforced by the observation that homologues of the *tatA*, *B* and *C* genes are located downstream of *xcpX* (PP1041, 1040, and 1039, respectively). Interestingly, the *P. putida* KT2440 genome sequence contains a second cluster of *tat* genes (PP5016–PP5018), suggesting that the former cluster is specially dedicated to the transport of UxpA and UxpB.

Considering the different genetic organization of the *xcp* gene clusters in *P. putida* and in *P. aeruginosa*, it seems likely that they have evolved separately⁴¹. This supposition was underscored by the low homologies between the Xcp proteins of the two species. Indeed, similar percentages of identity were found when the Xcp proteins of *P. putida* were compared with the secretion proteins of more distantly related bacteria such as *K. oxytoca*. Furthermore, the genes flanking the *xcp* clusters were different in both organisms, consistent with a different location on the genome.

A second, entirely different Xcp-related system is encoded on the genome of *P. putida* KT2440. This system was initially identified in *P. putida*

strain GB-1, where it is required for the secretion of a manganese-oxidizing factor^{18, 44}. The genes for this secretion system are located on two loci on the chromosome. One locus contains an operon of two genes, designated *xcmT1* (PP3423) and *xcmS* (PP3424), encoding proteins homologous to the pseudopilin XcpT and the integral membrane protein XcpS, respectively. Upstream of *xcmT1*, in the opposite orientation, is a gene *ltg*, encoding a lytic transglycosylase. Possibly, the product of this gene locally modifies the peptidoglycan layer allowing for the formation of a cell envelope-spanning macromolecular Xcm complex. The other locus contains a large operon, including the genes *xcmR*, *xcmQ*, *xcmT2*, *XcmT3* and *xcmX*⁴⁴. The genes *xcmR* and *xcmQ* encode homologues of the traffic ATPase XcpR and of the secretin XcpQ, respectively. The genes *xcmT2*, *xcmT3* and *xcmX* all three encode pseudopilins. Like XcmT1, XcmT2 and XcmT3 show a higher degree of homology to XcpT than to any of the other pseudopilins of *P. aeruginosa*. Furthermore, XcmX received its name because, like XcpX, it lacks the highly conserved glutamate at position +5 in the N-terminal hydrophobic stretch (see Section 2.2.1). For the rest, it does not show any additional homology to XcpX. Whereas XcpX is considerably longer than the other pseudopilins, XcmX is of normal length. Actually, XcmX rather shows some similarity to XcpT. In between *xcmR* and *xcmQ*, there are four genes (PP3479-PP3482), which do not show any homology to type II secretion genes, or actually, to any gene of known function. The putative PP3480, PP3481, and PP3482 proteins present one membrane-spanning segment each, whereas the putative PP3479 protein appears to be cytoplasmic. Considering the location of these genes in the *xcm* operon, the involvement of their products in the secretion of the manganese-oxidizing factor seems likely, although this has not been proven. Actually, only mutations in *xcmT1*¹⁸ and *xcmX*⁴⁴ have been described, showing a secretion defect. Additionally, a mutation in the *xcpA* gene disrupted the secretion of the manganese-oxidizing factor¹⁸, which is consistent with the identification of pseudopilins involved in the secretion process. Thus, the Xcm system is rather deviating from the classical type II secretion systems. Whereas the classical system contains five different pseudopilins, the Xcm system contains four, mutually related pseudopilins. Moreover, homologues of XcpP, XcpY and XcpZ appear to be absent in the Xcm system. Possibly, the unrelated genes PP3479-PP3482 replace them. Because of these deviations, we tend to classify the Xcm system in a sub-group different from the classical type II system, and we propose the name type IIb. Database searches revealed the presence of related type IIb systems, amongst others in *P. fluorescens* PfO-1, in *Ralstonia solanacearum*, which actually contains two copies of the system, one on the chromosome and the other on the megaplasmid, *Azotobacter vinelandii* and *Nitrosomonas europaea*.

Further analysis of the *P. fluorescens* PFO-1 genome revealed the presence of another type II secretion system, in addition to the type IIb system described above. In terms of homology and gene organization, this second system is most related to the *hxc* system of *P. aeruginosa* devoted to the secretion of low-molecular weight alkaline phosphatase (see Section 2.5.2). In *P. fluorescens*, the genes are organized in two clusters, one comprising homologues of *hxc*XYZQRS and of *lapA*, encoding low-molecular weight alkaline phosphatase (gene numbers Pflu1621-1613, respectively), and the other comprising homologues of *hxc*PUW (Pflu0069-0071, respectively). Within the clusters, the arrangement of the genes is the same as depicted for the *P. aeruginosa* genes in Figure 1. A homologue of *hxcV* was not found.

A BLAST search for homologues of XcpQ and XcpR in the genome database of *Pseudomonas syringae* pv. tomato at <http://www.tigr.org> revealed the presence of a typical type II secretion gene cluster with the gene annotations *gspEFGHIJKLMND* (corresponding to *xcpRSTUVWXYZ-gspN-xcpQ*) (gene numbers PSPTO3317-3307, respectively). A similar gene cluster was found in the genome of *P. syringae* pv. syringae. The products of these genes generally show the highest homology to the type II secretion proteins in *Xanthomonas* spp., where also the organization of the gene cluster is conserved. Remarkably, no gene in this gene cluster has been annotated as a *gspC* (*xcpP*) homologue. It should be noted, however, that the homology among the GspC/XcpP proteins of different species is very low. Furthermore, like GspC/XcpP, GspN is a bitopic inner membrane protein with a single N-terminally located putative transmembrane domain, and the homology of the GspN proteins of both *P. syringae* strains to other GspN-family members, such as PulN of *K. oxytoca*, is very poor. Therefore, we postulate that the gene, annotated as *gspN* in the *P. syringae* chromosome, actually represents the functional *gspC/xcpP* homologue. The gene downstream of the *gsp* cluster in both *P. syringae* strains putatively encodes a phospholipase/carboxyl esterase, which might well be a substrate of the Gsp secretion system.

Three additional XcpQ homologues were identified in the BLAST search of the *P. syringae* pv. tomato genome. PSPTO5128 is located in a cluster of genes involved in type IV pili biogenesis and represents a *pilQ* homologue. Considering its location in a cluster of genes involved in type III secretion and its homology to other secretins involved in type III secretion, PSPTO1389 is most likely involved in this secretion pathway. Like the *xqhC* gene of *P. aeruginosa* (see Section 2.5.3), PSPTO4851 is located in a cluster of *tad* genes and its product might therefore have a function in tight adherence. Similar *tad* genes were also found in the *P. fluorescens* PFO-1 genome.

3. AUTOTRANSPORTERS

3.1. The Autotransporter Mechanism

The autotransporter pathway probably represents the simplest secretion mechanism (for a review, see ref. [76]). Autotransporters are synthesized with an N-terminal signal sequence, which directs their transport across the inner membrane, presumably via the Sec machinery. The subsequent transport across the outer membrane does not require dedicated machinery, but is self-mediated by virtue of a C-terminal extension of the protein, the translocator domain. This domain presumably inserts into the outer membrane to form a pore, through which the functional domain, the passenger, is transported to the cell surface. It should be noted, however, that the autotransporter mechanism is dependent on the general insertion machinery for outer membrane proteins, of which Omp85 (PA3648 in *P. aeruginosa*) is a component¹⁶³. Hence, the name “autotransporter” represents an oversimplification of the actual transport mechanism. Two models for outer membrane transport have been proposed. In the original model, the passenger domain is transported in an unfolded state through a narrow pore formed by a single translocator domain^{86, 133}. This mechanism was supported by the observation that disulphide bond formation in an artificial passenger domain prevented translocation⁸⁷. More recently, however, limited translocation of disulphide bond-containing passengers fused to translocator domains was reported¹⁵⁹, suggesting that the channel is wider. Consistently, a recombinant translocator domain purified from the outer membrane of *E. coli* showed multimeric ring-like structures with a central cavity in electron micrographs¹⁶⁰. Hence, it was suggested that the passenger domains are translocated through the central channel of this multimeric structure. Recently, the crystal structure of the translocator domain of the neisserial autotransporter NalP was solved^{125a}. The structure revealed a β -barrel consisting of 12 antiparallel amphipathic β -strands that is filled by an N-terminal α -helix. This structure strongly supports the model that the passenger domain, which would normally be attached to the N terminus of α -helix, is transported through the barrel to the cell surface. Folding at the external surface could provide the driving force to thread the delineated passenger through the pore. In the passenger domain of the *Bordetella pertussis* autotransporter BrkA, a conserved domain was identified that might function as an intramolecular chaperone to effect folding at the cell surface¹²⁵. After transport, the passenger may remain anchored to the membrane via the translocator domain, or it may be released after autoproteolytic cleavage. Indeed, the passengers of many autotransporters contain a protease subdomain. However, this extracellular processing may also occur intermolecularly, even between different autotransporters, as was recently demonstrated in *Neisseria meningitidis*^{158a}. Perhaps,

the multimeric complexes reported by Veiga *et al.*¹⁶⁰ primarily play a role in such intermolecular processing.

Native passenger domains of autotransporters form a large and functionally diverse group of secreted proteins that are often implicated in pathogenesis (for a review, see ref. [75]). The first autotransporter studied in detail was the IgA1 protease of *Neisseria gonorrhoeae*¹³³. Other examples are the adhesins Aida-1 of *E. coli*⁸ and Hap and Hia of *Haemophilus influenzae*^{77, 146}, the serum-resistance protein BrkA of *B. pertussis*⁵³, the cytotoxin VacA of *H. pylori*¹⁴², and VirG of *Shigella flexneri*, which causes actin polymerization to promote intercellular spreading¹⁴⁹.

3.2. Autotransporters in *Pseudomonas*

The first and only autotransporter of *P. aeruginosa* studied in some detail is the esterase EstA¹⁶⁵. The enzyme activity was discovered in a lipase-negative deletion mutant of strain PAO1, which still showed extracellular lipolytic activity towards short-chain *p*-nitrophenylesters. Sequencing of the *estA* gene revealed that the protein is an autotransporter and that it belongs to a new family of lipolytic enzymes with a GDSLS active site motif¹⁶⁵. The enzyme is not processed at the cell surface and it remains firmly anchored to the outer membrane. BLAST searches revealed the presence of EstA homologues amongst others in the genome sequences of *P. aeruginosa* strain PA14, *P. putida* KT2440 (PP0418), *P. fluorescens* PfO-1, *P. syringae* pv. tomato, and *P. syringae* pv. syringae, indicating that it is an important protein for Pseudomonads.

Analysis of the genome sequence of *P. aeruginosa* PAO1 revealed the presence of only two additional putative autotransporter genes, PA0328 and PA3535¹⁰⁹. For comparison, we identified eight autotransporter genes in the genome of *N. meningitidis* strain MC58, which is three times as small as that of *P. aeruginosa*¹⁵⁸. The low abundance of autotransporter genes in *P. aeruginosa* may be related to the presence of efficient and versatile type II secretion systems in this organism. Like EstA (646 amino acid residues), the PA0328 protein (647 residues, including the signal sequence) is rather small for an autotransporter. Usually, autotransporters are larger proteins. The translocator domain of PA0328 shows high homology to that of EstA. The passenger domain is probably an aminopeptidase. It shows homology to known aminopeptidases, including the periplasmic alkaline phosphate-isozyme-conversion enzyme Iap of *E. coli*⁸¹. PA3535 (995 residues) belongs to a large family of serine-protease autotransporters, many members of which have been described in other *Pseudomonas* species, including *P. fluorescens* strain no. 33⁹³, *P. tolaasi*⁶⁸ and *P. brassicacearum*¹⁹. In those bacteria, but not in *P. aeruginosa*, these

serine-protease autotransporters are located in a cluster of genes involved in the type I-dependent secretion of protease and lipase. It should be noted that no proteolytic activity of these putative serine proteases has ever been demonstrated. The *P. brassicacearum* homologue, designated PspB, was suggested to be involved in autoaggregation¹⁹.

A BLAST search of the *P. putida* KT2440 genome sequence revealed two additional putative autotransporters, that is, PP1880 (730 amino acid residues) and PP3069 (825 residues). Both proteins are probably adhesins, since their highest similarity to proteins of known function was to the adhesins pertactin of *B. pertussis* and Aida-1 of *E. coli*, respectively. It is remarkable to find homologues of these virulence factors in a non-pathogenic strain. A homologue of PP1880 was found in the *P. fluorescens* PfO-1 genome sequences and two homologues were found in each of the *P. syringae* sequences. The *P. syringae* sequences contained also a homologue of PP3069. Finally, the BLAST search of the *P. putida* genome sequence revealed a short autotransporter-like gene (PP4057). The hypothetical translated product of the ORF is only 389 amino acid residues long and corresponds to the C-terminal translocator domain of autotransporters. No signal sequence could be discriminated at the N terminus. Hence, the protein, if actually produced *in vivo*, cannot be transported to the outer membrane to mediate the secretion of a non-associated passenger domain. Similar orphan translocator domains were found in the genome sequences of *P. fluorescens* and *P. syringae* pv. *syringae*.

BLAST searches on the genomes of *P. fluorescens* PfO-1, *P. syringae* pv. tomato DC3000 and *P. syringae* pv. *syringae* B728a yielded several additional putative autotransporters (data not shown), most of them belonging to the serine protease family of autotransporters. In the *P. fluorescens* genome, one putative autotransporter gene (Pflu4662) was identified that showed homology in the passenger domain to protein tyrosine phosphatases. In total, our BLAST searches revealed the presence of three complete putative autotransporters in the genome of *P. aeruginosa* PAO1, four in *P. aeruginosa* PA14, three in *P. putida* KT2440, seven in *P. fluorescens* PfO-1, nine in *P. syringae* pv. tomato DC3000 and eight in *P. syringae* pv. *syringae* B728a.

4. TWO-PARTNER SECRETION (TPS)

4.1. TPS Mechanism

The TPS system (for a review, see ref. [84]) is a two-step pathway, in which the substrate molecules, collectively designated TpsA, are synthesized in a precursor form with an N-terminal signal sequence and transported

across the inner membrane, presumably via the Sec system. Transport across the outer membrane is mediated by the second component of the TPS system, an integral outer membrane protein, generally designated TpsB. Usually, genes for the substrate molecule and the cognate TpsB component are organized in an operon. At first sight, the TPS systems seem to represent a variant of the autotransporter systems where the passenger domain is uncoupled of the translocator domain. However, there are some serious considerations to treat these systems as being different, rather than as subclasses of the same system: (a) There is no sequence homology between the translocator domains of the autotransporters and the TpsB components; (b) The TpsA components have to be targeted to their cognate TpsB component, whereas in the case of the autotransporters, the substrate is covalently attached to the translocator domain; (c) whereas in both systems transport can be driven by the extracellular folding of the substrate protein, it is conceivable that, in the autotransporter system, the first part of the passenger domain is brought to the cell surface by the insertion of the translocator domain into the outer membrane. In contrast, the energetic requirements for the initiation of transport of the TpsA component form a major conceptual problem. Nevertheless, there are also clear resemblances between the systems. For example, many, but not all, autotransporters and TpsA proteins are synthesized as precursors with an extremely long signal sequence, extended at the N terminus with ~25 amino acid residues^{76, 84}. The functional significance of this N-terminal extension remains to be established. Furthermore, many TpsA proteins and passenger domains of autotransporters appear to share a similar structural motif, that is, a long β -helix⁹⁰.

The TPS system is generally used for the secretion of very large proteins, varying in size from approximately 1,000 amino acid residues to over 6,000 residues. Their known functions so far are mostly adhesins, cytolytins or iron-acquisition proteins. Well-studied examples include the filamentous haemagglutinin FHA of *B. pertussis*, the haemolysin ShlA of *Serratia marcescens* and the high-molecular weight adhesins HMW1 and HMW2 of *H. influenzae*. The partner proteins are rather uniform in size, approximately 60 kDa. They form β -barrels with, rather unusual for outer membrane proteins, the N terminus exposed at the cell surface⁶⁹. The TpsB components of cytolytin secretion systems have a dual role. Apart from secreting their TpsA partner, they activate it by a non-covalent association with a phospholipid molecule⁷⁸. The secretion signal that is recognized by the TpsB component is encompassed in the N-terminal domain of the TpsA^{83, 143} and seems to be rather well conserved among different TpsA proteins⁸⁴. After transport to the cell surface, the TpsA proteins may undergo further proteolytic processing. In the case of FHA, the protease involved was recently identified as an autotransporter, designated SphB1²⁹.

4.2. TPS in *Pseudomonas*

To the best of our knowledge, no TPS-secreted proteins in any *Pseudomonas* spp. have been characterized to date. Nevertheless, analysis of the *P. aeruginosa* PAO1 genome sequence revealed the presence of five TpsA-TpsB couples, that is, PA0041-PA0040, PA0690-PA0692, PA2462-PA2463, PA4541-PA4540 and PA4625-PA4624, and one orphan TpsA, that is, PA4082^{30, 109}. Our own search (unpublished results) revealed one additional couple, that is, PA2542-PA2543.

PA0041 and PA2462 both showed up in our search when FhaB of *B. pertussis* was used as a probe for database mining. Therefore, they probably represent filamentous haemagglutinins. The polypeptides are 3535 and 5627 amino acid residues long, respectively (including the signal sequences), and show high mutual homology (61% identity in the aligned parts). The corresponding TpsB components show even 98% sequence identity, indicative of a very recent gene duplication. A highly related gene, that is, PSPTO3230, with 60% sequence identity was found in the *P. syringae* pv. tomato genome, and the cognate TpsA, PSPTO3229 (6,274 amino acid residues long), showed 36% identity to PA0041, suggesting that it is also a filamentous haemagglutinin. PA0041 was recently detected in the culture supernatant of quorum-sensing mutants of *P. aeruginosa*, but not in that of the wild-type strain¹²³. Since many proteases, such as elastase, are induced by quorum sensing, PA0041 (or its transporter) might be prone to proteolytic degradation in the wild-type strain.

PA0690 (4,180 amino acid residues), PA4082 (1,018 residues), PA4541 (1,417 residues) and PA4625 (2,154 residues) were picked up in our database screen when the adhesin HMW1 of *H. influenzae* was used as a lead. Hence, these proteins may function as adhesins as well. An ORF (PA0691) putatively encoding a transposase separates the PA0690 gene from its cognate *tpsB* gene (PA0692). This gene cluster is located adjacent to the *hxc* type II secretion gene cluster, dedicated to the secretion of low-molecular weight alkaline phosphatase (see Section 2.5.2). Homologues of this TpsA, transposase and TpsB were also identified in the *P. fluorescens* PFO-1 genome sequence, that is, Pflu1612 (4,179 amino acid residues), Pflu0687, and Pflu0688, showing 59%, 70% and 71% identity at the amino acid level, respectively.

The orphan TpsA, PA4082, is located within the *cupB* gene cluster, a gene cluster specifying the components of a chaperone/usheer system for the assembly of fimbrial subunits¹⁵⁶. Notably, the *fhaBC* genes of *B. pertussis* also are located in such a gene cluster¹⁰⁶. PA4082 is homologous (33% identity) to PA4541. Thus, perhaps, PA4082 is transported across the outer membrane via the cognate TpsB of PA4541, that is, PA4540. For PA4625, expression and secretion in vivo was recently reported¹²³. As for PA0041, the protein was only

detected in the culture supernatant of quorum-sensing deficient mutants and not in that of the wild-type strain.

In BLAST searches, the previously undescribed TpsA, PA2542 (1,221 amino acid residues) shows some similarity to PA2462 and to putative filamentous haemagglutinins from *Xanthomonas* spp. The protein appears to be highly conserved among *Pseudomonas* spp., since homologues were found in the genome databases from *P. putida* KT2440 (PP2893; 61% identity), *P. syringae* pv. *syringae* (P syr3898; 60% identity), and *P. syringae* pv. *tomato* (PSPTO2750; 59% identity). It was also present in the *P. fluorescens* PFO-1 genome, but there, the ORF was split into two parts, Pflu0670 and Pflu0671, possibly due to a sequence mistake. Also homologues of the TpsB partner of PA2542, that is, PA2543, were found in all these genomes. These proteins, PP2892, Pflu0669, P syr3899 and PSPTO2749, showed 68%, 70%, 68% and 68% identity, respectively, to the *P. aeruginosa* protein. Interestingly, in all these gene clusters, a gene encoding an acetyltransferase of the GNAT family was present (PA2891 in *P. aeruginosa*), suggesting that these TpsA proteins might be acetylated during their biogenesis.

Further searches in the *P. putida* KT2440 genome revealed one complete TpsA-TpsB couple, one orphan TpsA, and one orphan TpsB. The TpsA component of the complete couple, PP1449 (1,509 residues) showed significant similarity to the ShlA haemolysin of *S. marcescens*. The TpsB partner, PP1450, showed homology (34% identity) to the ShlA partner, ShlB. Interestingly, a homologue of PP1449 (73% identity) was previously found as the end of an ORF in the genome of *P. putida* WCS358, but at a different position, that is, downstream of the type II secretion *xcp* gene cluster (hence it was named *dpx*), which would correspond to PP1052 in the KT2440 chromosome⁴¹. Apparently, significant chromosomal rearrangements took place between the two strains. Homologues of the PP1449-PP1450 system were also detected in *P. fluorescens* PFO-1. These proteins, Pflu4788–Pflu4787, showed 37% and 44% identity, respectively, to the *P. putida* proteins.

The orphan TpsA of *P. putida*, PP0806, is 6,310 amino acids large. The C-terminus of this protein is identical to a previously described protein of *P. putida* KT2440 of 2,147 amino acids long. This protein, mus-20, was shown to be required for attachment of the bacteria to corn seeds⁵¹. Probably, a sequence mistake explains the difference in length. The protein shows also significant similarity to a much shorter (2,468 residues) *P. aeruginosa* protein, PA1874, discussed in Section 5.2.3.2. The orphan TpsB component, PP0573, showed homology to an orphan TpsB in *P. fluorescens*, that is, Pflu4360.

Analysis of the *P. syringae* pv. *tomato* chromosome revealed one additional orphan TpsA, that is, PSPTO3210. This protein, 992 amino acids large, is homologous to the TpsA PSPTO3229. The two genes are located close to

each other on the chromosome, separated by several IS elements, which may have played a role in a gene duplication event. Possibly, PSPTO3210 is transported across the outer membrane by the partner of PSPTO3229, that is, PSPTO3230.

Further analyses of the genomes of *P. fluorescens* and *P. syringae* pv. *syringae* did not reveal any additional Tps components. Thus, in total we found in *P. aeruginosa* six complete Tps systems and one orphan TpsA, in *P. putida* two complete systems, one orphan TpsA and one orphan TpsB, in *P. fluorescens* three complete systems and one orphan TpsB, in *P. syringae* pv. *syringae* one complete system, and in *P. syringae* pv. *tomato* two complete systems and one orphan TpsA.

5. TYPE I SECRETION SYSTEM

5.1 General Features

5.1.1. Secretion Apparatus. The type I secretion apparatus is composed of three distinct proteins. Each protein is present as a multimer in the complex. The ATP-Binding Cassette (ABC) protein is embedded in the cytoplasmic membrane and belongs to a superfamily of proteins that hydrolyse ATP to energize diverse biological transport processes³⁵. It is organized as a homodimer with six hydrophobic α -helical transmembrane segments in each monomer. This region of the protein is also called MSD for multi-spanning domain. Each monomer also contains a nucleotide-binding domain (NBD) located at the C terminus and facing the cytoplasm. This NBD contains the canonical Walker A and Walker B motifs, and the signature for the members of the ABC protein family, or C motif (LSGGQ), is found in between. The structure of one member of the family, that is, the MsbA lipopolysaccharide (LPS) transporter, has recently been solved²⁰. The ATPase activity of the ABC component involved in type I secretion is rather weak. For PrtD, which is involved in protease secretion in *Erwinia chrysanthemi*, the K_m of the ATP hydrolysis is 12 μ M and the V_m was around 1.5 μ mol ATP hydrolysed/mg of protein/hr, which is quite low⁴².

The membrane fusion protein (MFP) is anchored in the cytoplasmic membrane by one single transmembrane domain, and the main part of the protein (about 100 amino acids) protrudes into the periplasm and supposedly contacts the outer membrane¹³¹. A main characteristic of the protein is that it contains a coiled-coil region at its C terminus¹³⁰.

Classical outer membrane porins (see Chapter 19) are trimers with each monomer forming a β -barrel structure composed of amphipathic β -strands

allowing for outer membrane insertion and pore formation. The third protein of the type I system is an outer membrane protein, but the structure of TolC from *E. coli*, the prototypical member of this family, revealed an unprecedented feature⁹⁶. Although TolC is indeed trimeric, each subunit contributes four β -strands for the formation of a single 12-stranded β -barrel. Even more surprisingly, the major part of TolC consists α -helices, which form together a 100 Å long tunnel, continuing the β -barrel pore deeply into the periplasm.

5.1.2. Secretion Signal. The targeting of the secreted protein to its dedicated secretion apparatus is rather specific. The *B. pertussis* adenylate cyclase (CyaA), which is normally transported by the Cya type I secretion system, can be heterologously secreted by the *E. coli* α -haemolysin secretion system (Hly)¹¹¹. Both α -haemolysin and adenylate cyclase are large proteins belonging to the repeats in toxin (RTX) family of proteins. These proteins contain the motif GGxGxD, which is repeated 9–41 times. Another class of proteins transported by the type I system consists of proteases, like those found in *E. chrysanthemi*, or the alkaline protease (AprA) of *P. aeruginosa*. These are smaller proteins, with a size of about 40–50 kDa, which contain only 4–6 repeats. Also the secreted proteases could be transported to some extent by a heterologous apparatus. However, whereas proteases can be partially transported by an RTX toxin-dedicated type I system, RTX toxins are not secreted by protease-dedicated systems. This strict specificity is likely due to the nature and/or the size of the protein to be carried into the type I tunnel rather than to a strict targeting problem⁴⁷.

The type I-dependent exoproteins are synthesized with an uncleavable C-terminal secretion motif. Any exoprotein devoid of this signal is no longer secreted. Moreover, exchanging the C-terminal domain between type I-dependent proteins completely changed the specificity for the secretion apparatus. For example, replacement of the last 90 amino acids of AprA by the last 103 residues of HlyA redirected the hybrid alkaline protease to the Hly type I secretion system⁴⁷. The size of the C-terminal secretion signal may vary from 40 residues in the case of the protease to 80 residues in the case of the RTX toxins. There is no particular conserved sequence of amino acids in these signals. However, in the case of the RTX toxins, the presence of conserved secondary structure elements including α -helices was suggested²¹. In the case of the proteases, the signal is systematically ending with a DxxV motif, with a negatively charged residue preceding three hydrophobic ones⁶⁴.

In addition to RTX toxins and proteases, several other proteins are type I-dependent for secretion. Examples are the lipase produced by *P. fluorescens*⁴⁸ and the haem-binding protein (HasA) produced by *S. marcescens*³. In some cases, like for HasA, no GGxGxD motifs were found; thus, apparently, these repeats are not essential for type I secretion.

5.1.3. Molecular Model for Type I Secretion. The archetype type I secretion system is the Hly system from *E. coli*. In this case, the ABC and MFP proteins are HlyB and HlyD, respectively, whereas the outer membrane component involved is TolC. Interestingly, TolC has additional functions, since it is also a component of various drug efflux pumps, which have a similar overall organization. The secretion of the α -haemolysin (HlyA) has been studied in great detail, and the current model for this process is as follows. The C-terminal domain of HlyA interacts with the pre-formed HlyBD complex, which results in the recruitment of TolC via HlyD¹⁵⁴. The HlyD trimer may provide a cylinder of identical diameter as that of TolC channel, thus forming a continuous periplasmic secretion channel. However, the structure of the AcrB drug efflux pump was proposed to fit exactly on the TolC structure, independently of the AcrA protein that belongs to the MFP family¹²⁰. Therefore, we may expect that HlyB and TolC will directly fit and that the MFP only functions to capture TolC. The TolC–HlyD interaction might involve coiled-coil interactions, which are generally dynamic and may, in this case, be responsible for channel opening for HlyA transport. The energy for the transport should essentially derive from ATP hydrolysis via HlyB, even though reports are also referring to proton-motive force requirement at later stages of the secretion process⁹⁵. Finally, extrusion into the extracellular medium allows for Ca^{2+} binding to the GGxGxD sites, which contributes to acquisition of tertiary structure and, finally, to release of the toxin into the extracellular medium.

5.2. *P. aeruginosa* Systems

The type I secretion system in *P. aeruginosa* was initially discovered for the transport of alkaline protease (AprA). AprA is an important virulence factor, which contributes to tissue destruction. However, the PAO1 genome sequence revealed that additional type I systems are present within this strain.

5.2.1. The Apr System. The machinery for the secretion of alkaline protease in *P. aeruginosa* is composed of the three proteins AprD, E and F, corresponding to the ABC, MFP and outer membrane component of the type I system, respectively⁷⁰. The *apr* gene cluster contains two additional genes, *aprI* and *aprX*. The *aprI* gene encodes a specific inhibitor of alkaline protease⁴⁶. It is a signal peptide-containing protein, which is transported into the periplasm. Therefore, somehow, the alkaline protease might be exposed to the periplasm during transport in order to meet AprI. This observation is controversial with the established dogma that type I-dependently secreted proteins bypass the periplasm. In this respect, it may be interesting to note that the heat-stable enterotoxin has been reported to be secreted across the outer membrane via TolC after its transport across the inner membrane via the Sec system¹⁷⁵. Thus,

whereas the enterotoxin may enter the TolC channel in the periplasm, alkaline protease may occasionally escape from the channel in the periplasm, which would explain the need for the periplasmic inhibitor. Indeed, low levels of Colicin V (ColV) were detected in the periplasm during the secretion of the antibacterial peptide toxin ColV in *E. coli* via the type I system composed of CvaA, CvaB and TolC¹⁷⁷.

The *aprX* gene encodes a protein of unknown function, which was demonstrated, just like AprA, to be secreted into the extracellular medium via the Apr secretion machinery⁴⁵. In agreement with this observation is the fact that *aprX* is organized in a single operon together with *aprDEF*.

5.2.2. The Has System. The *P. aeruginosa* Has system is very similar to the one described in *S. marcescens*¹⁰³. It is required for transporting the haem-binding protein HasA into the extracellular medium. After HasA has bound haem, the HasA/haem complex is returned to the bacterial cell surface, where it binds to the outer membrane receptor HasR¹⁰². The *P. aeruginosa* *hasA* gene encodes a protein with 53%, 38% and 32% identity with the HasA proteins from *S. marcescens*, *P. fluorescens* and *Yersinia pestis*, respectively. On the chromosome, the *hasA* gene (PA3407) is located directly downstream of the gene encoding the HasR receptor, whereas the genes encoding the ABC (*hasD*), MFP (*hasE*) and outer membrane components (OpmM) are located downstream of *hasA*.

5.2.3. Other Systems. Analysis of the PAO1 genome¹⁴⁷ revealed at least two other systems, which could be referred to as type I secretion systems on the basis of sequence homology.

5.2.3.1. PA4142/PA4143/PA4144. This cluster encompasses the genes annotated PA4142 to PA4144. These genes encode an MFP protein (Prosite signature: PS00543), an ABC protein and an outer membrane protein (OpmK), respectively. The OpmK's highest homologue is CyaE, which is involved in the type I-dependent secretion of the calmodulin-sensitive adenylate cyclase of *B. pertussis*⁶⁵. This is a bifunctional protein, carrying both adenylate cyclase and haemolytic activities. PA4142 and PA4143 encode proteins with high similarities to CvaA and CvaB, respectively, of the ColV secretion system¹⁷⁷. Therefore, PA4142-4144 may encode a type I secretion system for an unknown *P. aeruginosa* exoprotein. Generally, the substrate of a type I system is encoded by a gene, located in the near vicinity of the genes encoding the secretion system. In this case, the gene downstream of PA4144 encodes a regulatory protein from the LysR family (48% homology with PtxR). The upstream gene, PA4141, is encoding a small protein, 99 amino acids long, of unknown function. Since this protein is small, just like ColV, it may be

transported by the system, but it is not possible to deduce this from the sequence.

5.2.3.2. PA1877/PA1876/PA1875. Another putative type I system might be encoded by the genes PA1877, 1876, 1875, which are homologous to the ABC, MFP and outer membrane proteins, respectively. Whereas PA1875 encodes a protein, OpmL, highly homologous to *P. aeruginosa* AprF, PA1877 encodes a homologue of the ExpD2 MFP protein from *Sinorhizobium meliloti*. In this bacterium, ExpD1 (ABC) and ExpD2 are required for the secretion of the ExpE1 protein, and all these *exp* gene products are directly or indirectly required for the biosynthesis and/or secretion of the exopolysaccharide galactoglucon (EPSII)¹¹⁸. Finally, PA1876 is encoding a protein highly homologous to a putative ABC transporter from *Rhodobacter capsulatus*. Directly downstream of PA1875, PA1874 encodes a large protein, which might be the substrate of this type I secretion system. However, sequence analysis does not clearly reveal the presence of glycine/aspartate repeats within the protein or a C-terminal domain characteristic of type I-dependent exoproteins. Sequence comparisons using the BLAST program revealed homologies with large-size surface proteins in other organisms, including a putative haemagglutinin/haemolysin from *R. solanacearum* and a putative surface adhesion protein from *P. putida* (PP0806; see also Section 4.2). Because of these homologies, and also because of the large size of the protein (2,486 residues), it seems more likely that the protein is secreted via a TPS system. However, since no *tpsB* gene is located in the vicinity, transport via the type I system remains a possibility, which should be investigated.

5.2.3.3. PA4974: Another TolC Homologue. A BLAST search with the AprF amino acid sequence as a probe against the PAO1 genome database revealed a protein (PA4974; OpmH) highly homologous to outer membrane proteins involved in type I secretion (e-value 3e-040). However, no ORFs encoding homologues of an ABC and a MFP could be found in the vicinity of this gene. In *E. coli*, TolC is involved in various type I secretion and drug efflux pump systems². Interestingly, *tolC* is also not located in the vicinity of ABC- and MFP-encoding genes, which is consistent with its role in multiple machineries. Thus, PA4974 could also have multiple functions in *P. aeruginosa* type I secretion systems and drug efflux pumps.

6. TYPE III SECRETION

The type III secretion system (TTSS), found in a wide range of animal- and plant-pathogenic Gram-negative bacteria, represents a powerful cytotoxic

weapon against the host cell. The contact and cross-talk between bacteria and their eukaryotic target cells lead to the injection of bacterial proteins across the bacterial membranes and the eukaryotic plasma membrane into the cytosol of the target cell. Those bacterial effector proteins subvert the host by diverting its signal transduction pathways, which control several essential biological processes. The archetype TTSS is the Ysc system of pathogenic *Yersiniae*, which delivers effector proteins, called Yops, into the host cells (for a review, see ref. [26]). Other Yops form pores in the host-cell membrane, through which the effector Yops are translocated. Additional proteins involved in *Yersinia* type III secretion include specific chaperones, generally designated Sycs, proteins that regulate the secretion process (YopN, TyeA, LcrG) as well as transcriptional regulators (VirF, YscM1, YscM2).

It is well established that the virulence of *P. aeruginosa* depends on its ability to synthesize and secrete proteins that aid its survival and replication in compromised hosts. In 1996, D.W. Frank and co-workers discovered that *P. aeruginosa* is endowed with a TTSS, allowing the delivery of a cytotoxin called ExoS^{58, 171}. Since then, two other cytotoxins (ExoT and ExoU) and one adenylate cyclase (ExoY) were also shown to be translocated by this machinery. In the following sections, we will review the current knowledge of the *P. aeruginosa* TTSS.

6.1. The Exo Effectors

Strains of *P. aeruginosa* have different combinations of genes encoding TTSS effectors and can be separated into two groups. The cytotoxic strains possess *exoU*, *exoT* and sometimes *exoY* (e.g., strain PA103 contains *exoU* and *T*), while invasive isolates possess *exoS*, *exoT* and often *exoY* (e.g., strain PAO1 contains *exoS*, *T* and *Y*)^{57, 168}. The products of these genes promote deleterious changes in host cells. For example, they inhibit phagocytosis by disrupting the actin cytoskeleton (ExoS and ExoT), they cause acute cytotoxicity and lung tissue damage (ExoU)^{55, 74}, and they elevate cellular cAMP levels (ExoY)¹⁷⁴. ExoS is a bifunctional enzyme that includes a GTPase-activating (GAP) function within its N-terminal domain⁶⁶ and a 14-3-3-dependent ADP-ribosyltransferase (ADPRT) activity within its C-terminal domain⁹⁴. In vivo, its GAP activity stimulates the reorganization of the actin cytoskeleton by inhibition of Rac and Cdc42 and stimulates actin stress fibre formation by inhibition of Rho⁹⁸. Intoxication of macrophages by ExoS could lead to an inhibition of the two major pathways of phagocytosis during *P. aeruginosa* infections, the type 1 immunoglobulin-receptor-utilizing pathway, which is mediated by Cdc42 and Rac; and the type 2 pathway, which utilizes the complement receptor and is mediated by Rho. ExoS ADP-ribosylates, among others, Ras, which inhibits the Ras-mediated signal transduction pathway⁶¹, and Rab5, one of the

Rab proteins involved in the early steps of the endocytic process⁷. Residues 51-72 of ExoS (termed membrane localization domain [MLD]) are necessary and sufficient for membrane localization within eukaryotic cells and are required only for the ADPRT activity¹²⁸. ExoT is a GAP for RhoA, Rac1 and Cdc42 *in vivo*⁹² and is an ADPRT, albeit with lower catalytic rates as compared to ExoS¹⁰⁵. ExoU is a lipase that requires activation or modification by eukaryotic factors¹³⁹.

Macrophages and epithelial cells respond differently to the *Pseudomonas* TTSS²⁴. Whereas lung epithelial cells showed significant changes in morphology, but not in viability, infected macrophages were efficiently killed. The TTSS dependency for rapid oncosis of macrophages and polymorphonuclear neutrophils was observed for several clinical isolates³². This may have implications on the pathogenesis of *Pseudomonas* infections. The disruption of lung epithelia may contribute to the bacterial penetration into subsurface tissues, while killing of macrophages may facilitate airways colonization and systemic dissemination.

Adherence to host cells is a crucial step in the initiation and establishment of bacterial infections and is a pre-requisite for type III secretion. Comolli and co-workers²⁵ showed that adherence of *P. aeruginosa* to MDCK cells, mediated by the specific interaction between type IV pili and asialo GM1, can contribute to the killing of epithelial cells by an ExoU-dependent mechanism. ExoS translocation into HeLa cells, studied by ADP-ribosylation of Ras, was shown to be dependent on Type IV pili¹⁴⁸.

The sequencing of the PAO1 genome¹⁴⁷ revealed a large genome (6.3 million base pairs) that could reflect an evolutionary basis allowing for bacterial adaptation and survival in diverse environments. Recently, an algorithm for the prediction of putative type III effectors was developed¹²⁹. This analysis was validated by the identification of 54 ORFs in the PAO1 genome, among which ExoS and ExoT. It is unlikely that all these 54 ORFs encode secreted proteins, but this collection may provide an enriched database for putative *P. aeruginosa* type III effectors. One could examine whether these putative effector genes are co-regulated with the TTSS genes (see Section 6.6). However, a negative result would not be conclusive, since the study of a new type III effector in the phytopathogen *R. solanacearum* revealed that it was not co-regulated with the TTSS regulon¹⁰¹.

6.2. The Psc Injectisome

The proteins that are required for secretion (i.e., the translocation across the bacterial envelope) were called Psc for *Pseudomonas* secretion components. The genes encoding those proteins are organized in two non-contiguous, but neighbouring, operons, which contain the *exsD-pscBCDEFGHIJKL* and



Figure 2. Genetic organization of the TTSS regulon in *P. aeruginosa*. Proved and postulated binding of the transcriptional regulator ExsA is shown.

pscNOPQRTSU genes, respectively (Figure 2). The *psc* genes are remarkably homologous to the *ysc* genes encoding the TTSS apparatus in *Yersinia* species (from 41% identity between *pscP* and *yscP* to 90% between *pscR* and *yscR*). The similarity is also observed at the level of the genetic organization in operons, which is rather well conserved, although the order and the direction of the operons is somewhat different. Moreover, a number of genes, including *psc/yscB*, *D*, *E*, *G*, *H*, *K*, *L*, *M* and *P*, are only present in the TTSS of *Yersinia* and *P. aeruginosa*¹¹, whereas some others (*psc/yscC*, *F*, *I*, *J*, *N*, *O*, *Q*, *R*, *S*, *T* and *U*) are found in all TTSS from both animal and plant pathogens, as well as in the flagellum export apparatus, which shows striking similarity to the TTSS (see below). Two other genes, now annotated *pcr3* and *4* (PA1701 and 1702, respectively), should be re-designated *pscX* and *Y*, respectively, because of their high similarity with the *yscX* and *yscY* genes (64% and 60% identity, respectively).

Only four genes, *pscC*^{113, 114, 171}, *pscJ*, *pscN*⁹¹, and *pscL*³⁴ have actually been demonstrated to be required for secretion in *P. aeruginosa*. The high level of conservation with their *Yersinia* homologues (78%, 83%, 89% and 75%, respectively) may indicate that they have a similar function. YscC belongs to the secretin family of outer membrane proteins. It forms a very stable ring-shaped multimeric complex with an external diameter of about 200 Å and an apparent central pore of 50 Å⁹⁷. The lipoprotein YscW (formerly called VirG) is required for efficient targeting of the YscC complex to the outer membrane, and very interestingly, while some data suggest that the *exsB* mRNA is not translated⁶⁷, its hypothetical product would present 50% of similarity with YscW. YscN could be the putative energizer of the TTSS machinery of *Yersinia*, since it displays similarity to the β-subunit of the bacterial F₀F₁ proton-translocating ATPase¹⁶⁷. No ATPase activity has ever been demonstrated so far for YscN, but a mutation in one of its consensus nucleotide-binding motifs (Walker box A) abolished Yop secretion. YscJ is a lipoprotein that was proposed to connect the inner and outer membranes¹¹⁷. YscC, YscN and YscJ homologues are present in all TTSS apparatus. Moreover, YscC and YscJ homologues were recovered when TTSS machinery, also called injectisomes, were purified from *Salmonella*¹⁰⁰ and *Shigella*^{14, 151}. The TTSS apparatus resembles the basal body of the flagellum (with which it shares about one third

of homologous proteins). The basal body is spanning the entire cell envelope, consisting of the peptidoglycan layer and the two bacterial membranes. It is topped by a needle-like structure exposed to the cell surface⁹⁹. The similarities between injectisomes and flagella are significant and suggest a common evolutionary origin, with the flagellum probably being the ancestor. YscF and its homologues from *Salmonella*, *Shigella* and enteropathogenic *E. coli* were identified as being the structural subunit of the needle^{14, 79, 100, 166}. It is thus reasonable to propose that this is also the case for PscF of the *Pseudomonas* injectisome. Such hypothesis is reinforced by the prediction of a coiled-coil structure at the N terminus of the PscF protein. Coiled-coil domains are involved in homotypic protein-protein interactions.

Two recent studies reported that a *dsbA* mutant of *P. aeruginosa*, lacking the periplasmic thiol:disulfide oxidoreductase DsbA, was defective in the secretion of type III effectors^{34, 71}. The use of various promoter fusions suggested that DsbA was required for the expression of TTSS genes⁷¹. We favour another hypothesis, that is, that some of the Psc components require DsbA to be active. This might certainly be the case for the secretin PscC as DsbA dependency was demonstrated for its homologue YscC⁸². The lack of TTSS regulon expression observed in *dsbA* mutant could be explained by feedback repression as observed in secretion mutants.

6.3. Chaperones

Before being secreted, proteins are stored in the cytoplasm where they are associated with small proteins called TTSS chaperones. Panoply of function has been proposed for the members of this family, for example, stabilization of their cognate exoprotein, maintenance of a secretion-competent state by preventing aggregation, targeting to the secretion apparatus and setting a hierarchy on secretion, and participation in the regulation of the transcription of TTSS regulon^{52, 127}. However, their precise role is still mysterious. Although TTSS chaperones do not exhibit sequence similarities, they share common features, such as a small size, an acidic pI, and a predicted amphiphilic α helix in their C-terminal part. Several classes of TTSS chaperones are distinguished¹²⁷. Three of them are presumably present in *P. aeruginosa*. The members of the first class are strictly associated with one effector, those of the second class with the two proposed translocators (see further), while the third class consists of two chaperones serving a single substrate. Specific *Pseudomonas* chaperone for ExoU (SpcU) is the only chaperone of *P. aeruginosa*, of which the role has been examined. SpcU is required for the efficient secretion of the cytotoxin ExoU⁵⁶, and it binds to the first 123 residues of ExoU. It was proposed that Orf1, encoded just upstream of the *exoS* gene, could serve as a specific chaperone for ExoS¹⁷². Orf1 presents all the known

characteristics of TTSS chaperones and might be re-named SpcS. No gene putatively encoding a chaperone could be identified in the vicinity of the two other effector genes, *exoY* and *exoT*. However, it should be noted that in the *Yersinia* system also not all effectors do have dedicated chaperones. PcrH, which is encoded within the same operon as the translocators PopB and PopD is probably their associated chaperone, since an interaction between these three proteins was demonstrated¹. Finally, Pcr2 (which could be re-named SpcN) and PscB could possibly serve PopN, since they are homologous to SycN and YscB, respectively, which together function as chaperones for YopN in *Yersinia*³⁶.

Although there has been some controversy about the nature of the secretion signal that is recognized by the TTSS, notably whether it is located in the protein or in messenger RNA²⁶, the overall consensus is that the N-terminal region of the effectors is involved in targeting. C-terminal truncations of ExoS and internal deletions were constructed, and these experiments revealed that truncated proteins containing the 99 first residues were secreted¹⁷¹. Even though ExoS proteins with amino-terminal deletions were undetectable by Coomassie staining of SDS-polyacrylamide gels or immuno-revelation, measurement of their ADP-ribosyltransferase activity allowed their intracellular detection with little or no activity in the supernatant. N-terminal residues are thus required for ExoS secretion.

6.4. Secretion Control

In *Yersinia*, type III secretion is induced under low-Ca²⁺ conditions or upon contact of the bacteria with eukaryotic target cells. In *P. aeruginosa*, the production of ExoS is known to be inducible by chelation of divalent cations¹⁵⁵. Furthermore, the TTSS regulon expression could be induced in vitro by chelation of Ca²⁺ ions in the culture medium⁵⁸. Serum in tissue culture medium was also shown to trigger TTSS secretion in vitro¹⁵⁷. Finally, contact with eukaryotic cells induces effector translocation as shown for ExoS upon infection of Chinese hamster ovary (CHO) cells with *P. aeruginosa*¹⁵⁷.

In the *Yersinia* TTSS, three proteins called YopN, TyeA and LcrG are involved in the control of Yop release. It is generally proposed that YopN could function as a sensor that could form a plug together with TyeA and LcrG to block the secretion channel. This is based on the observation that *yopN*, *tyeA* and *lcrG* mutants secrete Yops in the presence of Ca²⁺. Secretion control in *P. aeruginosa* might be similarly exerted. PopN, a YopN homologue, was recovered in culture supernatant upon TTSS-inducing conditions¹⁷³. An *lcrG* homologue, *pcrG*, is present in the *Pseudomonas* genome. Finally, screening for genes required for *P. aeruginosa* cytotoxicity led to the isolation of a mutation in *pcr1* (PA1699), a gene homologous to *tyeA*⁹¹.

6.5. The Translocators PopB, PopD and PcrV

The translocators PopB, PopD and PcrV¹⁷³ are required for the transport of effectors across the eukaryotic cell membrane, presumably by forming a pore. In the various TTSS, translocators are the only secreted proteins that have hydrophobic domains, suggesting that they could interact with membranes. Expression of the *Pseudomonas pcrGVHpopBD* operon in a translocator-deficient strain of *Yersinia* resulted in complete complementation of the translocation defect, demonstrating that the *Pseudomonas* proteins do have the same role⁶⁰.

While the secretion of ExoS into the external milieu was not affected by a *popB*, *popD* or *pcrV* mutation, its translocation into the host cell was impaired^{148, 157}. Translocation of ExoY could be monitored by cAMP accumulation in and morphological changes of CHO cells, which indicated that ExoY translocation is PopD-dependent¹⁷⁴. Moreover, a mutant of the cytotoxic strain PA103 with a transposon insertion in *pcrG*, the first gene of the translocation operon, was defective not only in the in vitro secretion of PopB and PopD, but also in ExoU-mediated cytotoxicity towards epithelial cells and macrophage killing, while huge amounts of ExoU were still secreted in vitro^{74, 91}.

Pseudomonas aeruginosa exerted a contact-dependent, lytic effect on infected erythrocytes that required the PcrV, PopB and PopD proteins^{33, 60}. The size of the pore formed within the erythrocytes was between 2.8–3.0 nm as estimated from osmoprotection experiments⁶⁰ and from the use of fluorescent dyes³³. The pore forming-activity of the translocators leads to death of infected macrophages, which are rapidly surrounded by numerous motile bacteria, a chemotaxis-related phenomenon termed pack swarming³³.

The contribution of PcrV to the translocation process was confirmed in cytotoxicity assays in a macrophage cell line and in an acute lung infection model¹⁴¹. Both infection models showed the requirement of PcrV for effector translocation. More interestingly, antibodies specific for PcrV mediated protection against a lethal infection, lung injury and cellular cytotoxicity. The mechanism of antibody-mediated protection involves an inhibition of type III effector translocation into alveolar macrophages and cell lines. Of greater importance is the possibility that an effective vaccine can now be designed to combat the many chronic forms of disease caused by *P. aeruginosa*⁵⁹.

6.6. Transcriptional Regulation

ExsA, a member of the AraC/XylS family of transcriptional regulators, functions as a central regulator of the TTSS in *P. aeruginosa*, like its nearest homologue, VirF of *Yersinia*. ExsA activates transcription by binding specifically to promoters upstream of *exsCBA*, the *trans*-regulatory locus^{34, 80, 114, 170},

exsD-pscBCDEFGHIJKL, one of the two operons encoding components of the secretion apparatus^{80, 170} (Figure 2), *exoS* and its putative chaperone-encoding gene *spcS* (*orfI*)^{80, 169, 172} (Figure 2). DNase I footprint analysis of the promoter regions bound by ExsA revealed a protected consensus sequence, TxAAAxA, located 51 or 52 bp upstream of the transcriptional start site⁸⁰. Since ExsA consensus sequences are present in the following promoter regions with appropriate spacing, and since the corresponding secreted products were missing in *exsA* mutant strains, one could definitely propose that ExsA binds upstream of the *exoT* gene^{31, 171}, *exoU*⁵⁵, *popNpcrIspcNpscXYpcrD* (the “control release” locus), *pcrRGVHpopBD* (the translocation operon)^{31, 173} (Figure 2) and *exoY*¹⁷⁴. The promoter of the second secretion gene operon, *pscNOPQRSTU*, also contains an ExsA-binding site. Thus, the central transcriptional regulator ExsA co-ordinately controls all members of the TTSS regulon⁵⁸ (Figure 2). Transcriptome analysis of an *exsA* mutant or of the wild-type strain upon over-expression of *exsA* confirmed the ExsA dependency of all known TTSS genes, although the effect on the *exsD-pscBCDEFGHIJKL* operon was only marginal¹⁶⁹. This microarray analysis resulted also in the identification of a novel TTSS activating signalling pathway, dependent on cAMP and the cAMP-binding protein Vfr. A model in which cAMP and Vfr act upstream, or at the level of ExsA was proposed to link cAMP signalling and sensing of host environment¹⁶⁹ (Figure 3). Finally, ExsC may play a role in the translation or stability of *exoS*, while the untranslated *exsB* messenger facilitates either the stability or the translation of *exsA*⁶⁷.

In addition to the positive control exerted by ExsA, a negative regulatory loop has been postulated based on the observation that *exoS* gene expression was repressed by Ca^{2+} or by mutations within genes encoding the components of the secretion apparatus^{157, 171}. This feedback mechanism does not occur in secretion mutants upon contact with target cells. In this case, *exoS* is expressed and synthesized, but not translocated, while it is repressed in low Ca^{2+} conditions¹⁵⁷. Recently, ExsD was shown to be an intracellular negative regulator that could function as an ExsA anti-activator¹¹⁴. One potential mechanism to explain the ExsD-exerted control involves a competition between a secreted product and ExsD for binding a chaperone (Figure 3).

6.7. TTSS in Other *Pseudomonas* Species

One of the exciting surprises of the discovery of TTSS was that a variety of phytopathogenic bacteria do have and use TTSS for pathogenesis. *P. syringae* infects a wide range of susceptible plants in which it causes bacterial speck, and its TTSS is also essential for the elicitation of a plant defence reaction called the hypersensitivity response in non-host plants (for a review,

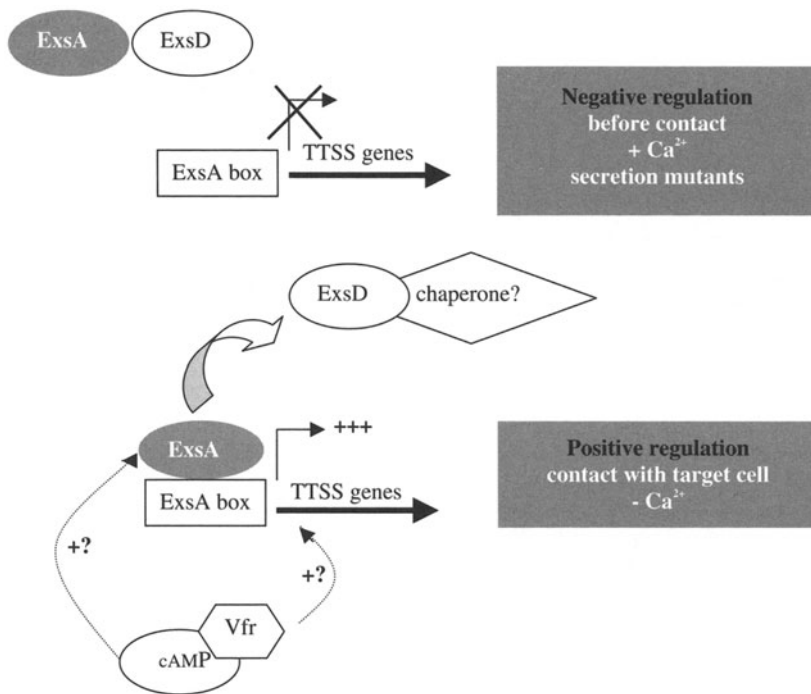


Figure 3. Model for the regulation of the transcription of TTSS genes. ExsA is the central transcriptional activator, which binds to the ExsA box in the promoter regions of all members of the TTSS regulon. Before contact with eukaryotic target cells in the presence of Ca^{2+} or in secretion mutants, ExsD prevents binding of ExsA to the ExsA boxes. When secretion is activated, chaperones are released from the secreted proteins and accumulate in the cytoplasm. They might bind ExsD, thus liberating ExsA and allowing ExsA to bind to the ExsA boxes and, thereby, to activate transcription of TTSS genes. Additional control is exerted by the Vfr–cAMP complex, which might activate transcription of the TTSS genes either directly, or indirectly by activating transcription of *exsA*.

see ref. [27]). The bacterial genes required for those two processes have been called *hrp* (hypersensitive response and pathogenicity). This secretion system is responsible for the assembly of the Hrp pilus⁸⁵ and is thought to deliver effector proteins, which are designated as Avr (avirulence) or Hop (Hrp-dependent outer proteins)¹²⁹, directly into the host cell.

A careful analysis of the genome of *P. putida* KT2440, a metabolically versatile saprophytic soil bacterium¹²¹, revealed that the only TTSS homologues present are those of the flagellar system. Thus, the TTSS is clearly absent in this bacterium, which has been certified as a biosafety host for the cloning of foreign genes and for biotechnological applications.

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CHEMOTAXIS IN PSEUDOMONADS

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1. INTRODUCTION

All *Pseudomonas* species are motile by one or more polar flagella and are highly chemotactic. Chemotaxis and motility have been implicated in virulence in *Pseudomonas aeruginosa*²¹, and are important for plant root associations in *Pseudomonas fluorescens*¹⁷. The chemotaxis machinery has not been studied in detail in any *Pseudomonas* species and the range of attractants and environmental conditions to which Pseudomonads can respond behaviorally remains largely unexplored. However, the availability of four *Pseudomonas* genome sequences has allowed the identification of numerous potential chemotaxis genes. Experiments in *P. aeruginosa* and *Pseudomonas putida* indicate that the general chemotaxis machinery present in these organisms is similar to that of the well-studied enteric bacteria *Escherichia coli* and *Salmonella*. The vast array of chemotaxis and receptor genes present in the *Pseudomonas* genomes suggests that chemotaxis may be more complex and sensory transduction may be more versatile in the Pseudomonads than in enteric bacteria. This chapter will

focus primarily on the information gleaned from the complete genome sequences of *P. aeruginosa* PAO1⁷⁴, *P. putida* KT2440⁵⁰, *Pseudomonas syringae* DC3000 (ref. 8a) and the unfinished *P. fluorescens* PFO1 sequences (http://www.jgi.doe.gov/JGI_microbial/html/index.html), correlating available functional data whenever possible.

2. THE *E. COLI*/SALMONELLA PARADIGM FOR SENSORY SIGNAL TRANSDUCTION IN CHEMOTAXIS

Flagellated bacteria and archaea have chemotactic signal transduction complexes that allow them to sense and swim toward microenvironments that provide optimal conditions for their growth and survival. Chemotaxis is mediated by a sophisticated “two-component” or histidine kinase phospho-signaling pathway^{72, 73}. In the model organism *E. coli*, a set of six chemotaxis proteins acts in concert with five receptors known as chemotaxis transducers or methyl-accepting chemotaxis proteins (MCPs). MCPs exist as homodimers that are physically associated with a dimeric CheW protein linker to a CheA protein dimer. MCPs act as primary chemoreceptors for some stimulants, and with others, act as secondary receptors that bind the specific receptor proteins. Each MCP has a sensing module, typically located in the periplasm, and a signaling module in the cytoplasm. Upon binding an attractant, a MCP undergoes a conformational change that initiates sensory signal transduction by altering the histidine kinase autophosphorylation activity of CheA (Figure 1). CheA-P is a phosphodonor for the response regulator protein CheY. The cytoplasmic CheY-P interacts with the rotational switch protein FliM, of the flagellar motor, causing a change from the default counterclockwise (CCW) rotation to clockwise (CW) rotation. During CCW rotation, the flagella form a bundle that allows the cell to swim smoothly in a single direction. When CheY-P interacts with FliM, causing the flagella to rotate in the CW direction, the bundle comes apart, and the cell tumbles, randomly changing direction. Thus, the phosphorylation status of CheY determines whether the cell spends more time smooth swimming or tumbling. When cells swim up a gradient of attractant, they modulate their behavior by spending more time smooth swimming than tumbling, thus making progress in the direction of the higher concentration of attractant. In addition to the intrinsic dephosphorylation activity present in CheY, CheZ controls its dephosphorylation. An adaptation system involving the methylation and demethylation of MCPs at specific glutamate residues allows the bacterium to continuously detect further changes in its environment. MCP methylation counterbalances the effect of attractant binding and resets

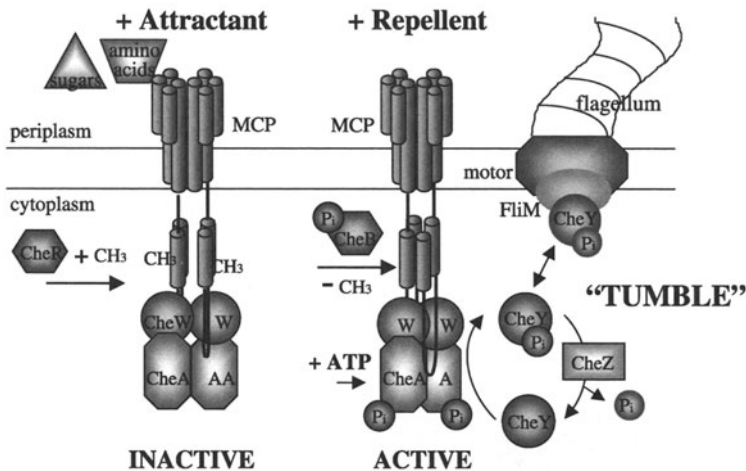


Figure 1. The chemosensory signal transduction pathway in *E. coli*. The chemotaxis machinery of unstimulated cells operates at an equilibrium between “plus attractant” and “plus repellent” states. Reprinted with permission from the American Society for Microbiology.

the signaling activity of the receptors regardless of the continued presence of stimulus. The CheR methyltransferase introduces methyl groups onto conserved glutamate residues and the CheB methylesterase, which is activated by phosphotransfer from CheA-P, removes the methyl groups.

3. CHEMOTAXIS GENES IN *PSEUDOMONAS* GENOMES

Experiments in *P. aeruginosa* and *P. putida* have demonstrated that these organisms have chemotaxis genes that are homologous to those present in *E. coli* and *Salmonella*. Chemotaxis (*che*) genes are located in a single gene cluster in *E. coli* and *Salmonella*⁷³. Genome sequence analysis has revealed the presence of multiple chemotaxis gene clusters in many bacterial genomes, including all four sequenced *Pseudomonas* chromosomes. Multiple copies of *cheA*, *cheY*, *cheW*, *cheB*, and *cheR* gene homologues are seen. The possible contribution of multiple sets of chemotaxis genes has been explored in the α proteobacteria *Sinorhizobium meliloti*, *Rhodobacter sphaeroides*, and *Caulobacter crescentus*, and in the γ proteobacterium *Vibrio cholera*. *S. meliloti* and *C. crescentus* each have two sets of chemotaxis genes^{26, 53}, and *R. sphaeroides* has three sets of chemotaxis genes⁶⁰. A single set of

chemotaxis genes clearly controls flagella-mediated chemotaxis in *S. meliloti* and *C. crescentus*^{68, 77}. However, the situation appears to be much more complex in *R. sphaeroides*. Three of the CheAs and three CheYs are essential for a normal *R. sphaeroides* chemotactic response, so clearly these proteins are not redundant⁶⁰. In addition, two CheBs, two CheRs and three CheWs are required for *R. sphaeroides* chemotaxis. Each homologue appears to play a unique role in chemotaxis although some partial overlap of functions were identified⁶⁰. *V. cholera* has three sets of chemotaxis genes, just one of which is essential for chemotaxis²⁷.

There are at least four possible functions for the extra sets of chemotaxis genes that are present in many bacteria. First, multiple sets of Che proteins might function together to effect optimal flagella-mediated chemotactic behavior. This seems to be the case in *R. sphaeroides*^{60, 64}. Second, extra sets of chemotaxis proteins might mediate chemotaxis in different environments, such as an animal host, but as yet, no evidence exists for this possibility. Third, extra sets of Che proteins might control cellular motors other than the flagellar motor. Indeed, chemosensory systems that control type IV pilus-dependent motility have been identified in *Myxococcus xanthus* (for social motility)^{79, 81}, *Synechocystis*, and *P. aeruginosa*^{6, 16}. Finally, additional sets of chemotaxis-like proteins might be used for other sensory-response systems that result in the activation or repression of gene expression. For example, Che homologues in *M. xanthus* have recently been shown to control expression of developmental genes by regulating a sigma 54-dependent transcriptional activator protein⁴².

The following section describes the Che-like gene clusters identified in the four *Pseudomonas* genomes and the available functional data.

3.1. Chemotaxis Genes in Clusters I and V are Required for Chemotaxis

General chemotaxis defects map to Clusters I and V in *P. aeruginosa* and *P. putida*, indicating that these genes, which are homologues of the *E. coli*/*Salmonella* chemotaxis genes, are required for chemotaxis. In each of the four *Pseudomonas* genomes, Cluster I encodes five of the six chemotaxis proteins found in enteric bacteria (CheA, CheB, CheW, CheY, and CheZ), and two proteins with some sequence similarity to *E. coli* flagellar motor components, MotA and MotB (Figure 2a). Prior to the sequencing of the *Pseudomonas* genomes, Cluster I was completely cloned and sequenced from *P. putida* PRS2000 and *P. aeruginosa* PAO1^{18, 39}. A *P. putida* *cheA* mutant was generally nonchemotactic and exhibited smooth swimming behavior under all conditions tested¹⁸. *P. aeruginosa* *cheA*, *cheB*, *cheW*, *cheY*, and *cheZ* mutants were nonchemotactic, although they were fully motile^{24, 39}. The *P. aeruginosa* *cheA*,

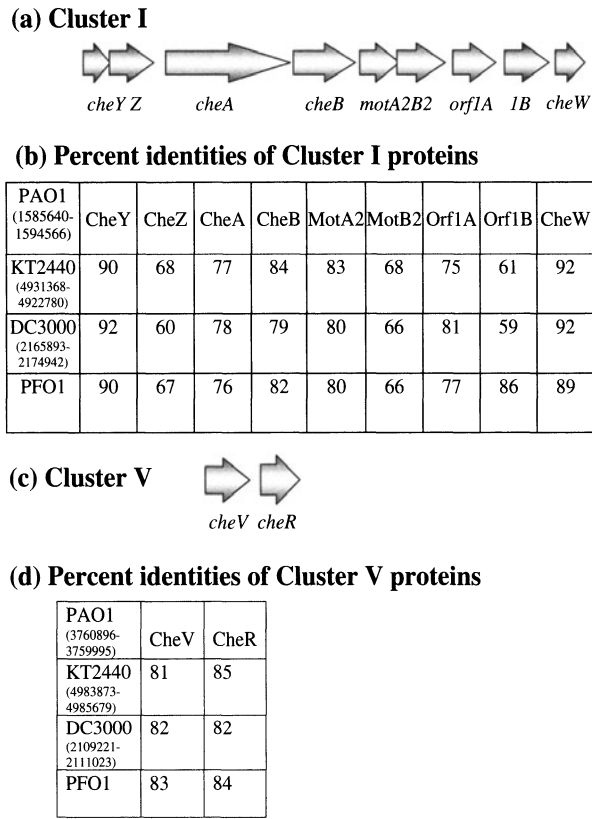


Figure 2. Clusters I and V in Pseudomonads. (a) Cluster I is present in all four sequenced *Pseudomonas* genomes. The gene order is identical in all cases. Flagellar biosynthesis genes are located upstream of Cluster I in all four strains. The downstream genes differ in each strain. (b) Percent amino acid identities for each of the Cluster I proteins relative to the PAO1 deduced amino acid sequence. (c) Cluster V is present in all four strains and is located approximately 50-kb downstream of Cluster I in the *P. syringae* DC3000 and *P. putida* KT2440 genomes. In *P. aeruginosa* PAO1 and *P. syringae* DC3000, genes upstream of Cluster V encode proteins with similarity to flagellar biosynthesis genes; downstream genes differ in each strain. (d) Percent amino acid identities of each of the Cluster V proteins relative to the PAO1 deduced amino acid sequence. The position of each cluster in a particular genome is given in parentheses under each strain name.

cheW, and *cheY* mutants had smooth swimming behavior; and the *cheB* and *cheZ* mutants changed direction excessively^{39, 44}. It is worth noting that *cheZ* homologues do not appear to be present in the α proteobacteria. These bacteria typically have multiple CheY homologues, some of which apparently serve as phosphate sinks to limit the phosphorylation of the motor-binding form of CheY^{59, 64, 69}.

cheR was not found in Cluster I, but is located in a 2-gene cluster (*cheVcheR*), designated Cluster V (Figure 2c), in all four sequenced *Pseudomonas* genomes. A *P. aeruginosa cheR* mutant was nonchemotactic and changed direction much less frequently than the wild type³⁹. All of the characterized *P. aeruginosa* and *P. putida* mutant phenotypes were similar to those in the corresponding enteric mutants, suggesting a similar mechanism of signal transduction. CheV is a two-domain protein that has an N-terminal CheW-like domain and a C-terminal two-component receiver (CheY-like) domain²⁵. *cheV* genes have been identified in various bacteria, including *Bacillus*, *Campylobacter*, *Salmonella*, and *Vibrio*, and in all four *Pseudomonas* genomes. In *Bacillus subtilis*, CheV is required for adaptation to attractants³⁷, but its role in *Pseudomonas* chemotaxis has not been studied.

Inactivation of the *motA* homologue in Cluster I of *P. aeruginosa* resulted in cells that were motile in liquid culture but that formed small dense colonies on swarm plates, and a *motB*-homologue mutant appeared to form normal swarms³⁹, suggesting that additional *motAB* homologues may be present on the *P. aeruginosa* chromosome. A search of the *Pseudomonas* genomes identified an alternative *motAB* pair with higher amino acid sequence similarity to *E. coli motAB*. The function of the *motAB* homologues in Cluster I therefore remains unclear. Three additional ORFs of unknown function are present in Cluster I. Based on mutagenesis studies, none of these ORFs appeared to be required for chemotaxis in *P. aeruginosa*³⁹.

3.2. Chemotaxis-Like Genes in Cluster II

Cluster II is present in *P. aeruginosa* PAO1, *P. syringae* DC3000, and *P. fluorescens* PF01. The gene organization differs slightly in *P. aeruginosa* (Figure 3a, b), and the context of Cluster II differs in each of the three chromosomes. This cluster includes homologues of *cheA*, *cheB*, *cheW*, *cheY*, and *cheR* as well as two MCP-like genes. Overall identities among the genes in the three strains are relatively low (Figure 3c).

In *P. aeruginosa* and *P. putida*, all screens for general chemotaxis mutants have identified genes in Clusters I and V only^{18, 39, 75}, so obviously genes in other clusters do not compensate for the Cluster I mutations, that is, they do not encode redundant functions. It appears that the Cluster II genes play, at most, a minor role in chemotaxis in *P. aeruginosa*, and may instead participate in some other environmental sensory transduction pathway. Of the *che* genes present in Cluster II, only the inactivation of the Cluster II gene *cheB2* resulted in a slight chemotaxis defect in *P. aeruginosa*²⁴. Overexpression of *mcpA*, *mcpB*, *cheA2*, *cheB2*, and *cheW2* (but not *cheY2*) in *E. coli* K-12 interfered with the normal chemotactic response, indicating that

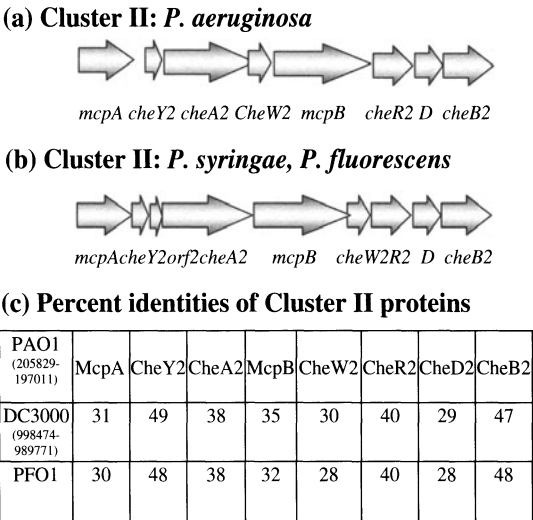


Figure 3. Gene organization of Cluster II. Cluster II is present in *P. aeruginosa* PAO1, *P. syringae* DC3000, and *P. fluorescens* PF01, but not *P. putida* KT2440. (a) The gene order of Cluster II in *P. aeruginosa* PAO1. (b) The gene order of Cluster II in *P. syringae* DC3000 and *P. fluorescens* PF01. The locations of *mcpB* and *cheW2* are opposite that in *P. aeruginosa* PAO1. The context of the Cluster II genes differs in each *Pseudomonas* genome. (c) Percent amino acid identities of each of the Cluster II proteins relative to the PAO1 deduced amino acid sequence. The position of a cluster in a particular genome is given in parentheses under the strain name.

these proteins can compete with the *E. coli* Che proteins involved in the signal transduction pathway²⁴. Recent work demonstrated that Cluster II genes are induced during stationary phase and further regulated by quorum sensing. These genes may be involved in attachment and biofilm formation^{35, 63}. It is interesting to note that *P. putida* KT2440 does not have a Cluster II, and this may reflect differences in its environmental niche.

3.3. Chemotaxis Genes in Cluster III

Cluster III, which is present and identical in gene order in all four *Pseudomonas* genomes, contains two *cheW*-like genes, a *cheR*-like gene, a *cheB*-like gene, a hybrid *cheA-cheY* gene, and a MCP-encoding gene (Figure 4). Genes have been named “wsp” based on studies carried out in *P. fluorescens* and *P. aeruginosa* in which a “wrinkly spreader phenotype” (describing an alternative colony morphology) has been identified^{11, 70}. A GGDEF-type response regulator^{36, 58} was shown to be required for the wrinkly spreader

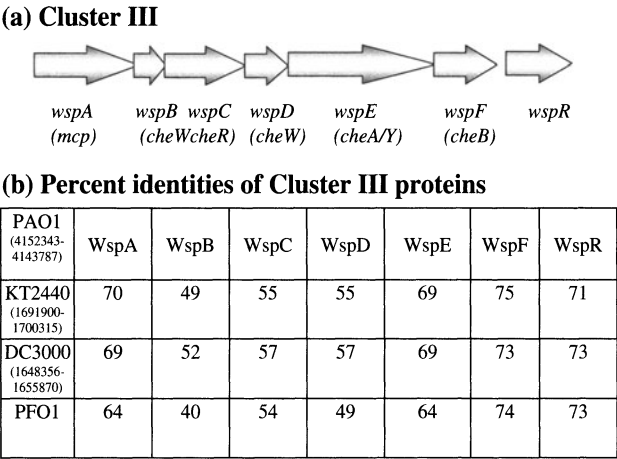


Figure 4. Cluster III *che*-like genes. (a) The organization of Cluster III. The gene order is identical in all four strains. Homologous *che* genes are indicated in parentheses. (b) Percent amino acid identities of each of the Cluster III proteins relative to the PAO1 deduced amino acid sequence. The position of a cluster in a particular genome is given in parentheses under each strain name.

phenotype in *P. fluorescens* and was designated *wspR*⁷⁰. The WspR homologues PleD, CelR2, and AgfD were shown to participate in the signal transduction pathway of stalked-cell differentiation in *C. crescentus*³⁶, regulation of cellulose synthesis in *Rhizobium leguminosarum* bv. *trifolii*⁴, and the production of aggregative fimbriae in *Salmonella typhimurium*⁶², respectively. *wspF* (the *cheB* homologue) and *wspR* mutants of *P. aeruginosa* formed wrinkled aggregative colonies, and suppressors of a *wspR* mutant mapped to the *wspA*, *wspB*, *wspC*, *wspD*, *wspE*, and *wspR* genes¹¹, indicating that the Cluster III genes are involved in cell aggregation.

3.4. Chemotaxis Genes in Clusters IV

Cluster IV is present in all four *Pseudomonas* genomes, but *P. aeruginosa* has four extra genes (*pilK*, *chpB*, *chpD*, *chpE*) not found in the other strains (Figure 5). *pilG* and *pilH* are *cheY* homologues, *pilI* and *chpC* are *cheW* homologues, *pilK* is a *cheR*-like gene, and *chpB* is a *cheB* homologue. In addition, *pilJ* encodes a MCP and *chpA* encodes a complex hybrid protein with *cheA* and *cheY* domains.

The genes present in Cluster IV are involved in pilus-mediated twitching motility in *P. aeruginosa*¹⁶. This form of flagella-independent surface motility⁴⁵ allows bacteria to rapidly colonize wet surfaces and has been shown to be involved in fruiting body formation in *M. xanthus*⁸⁰ and biofilm formation

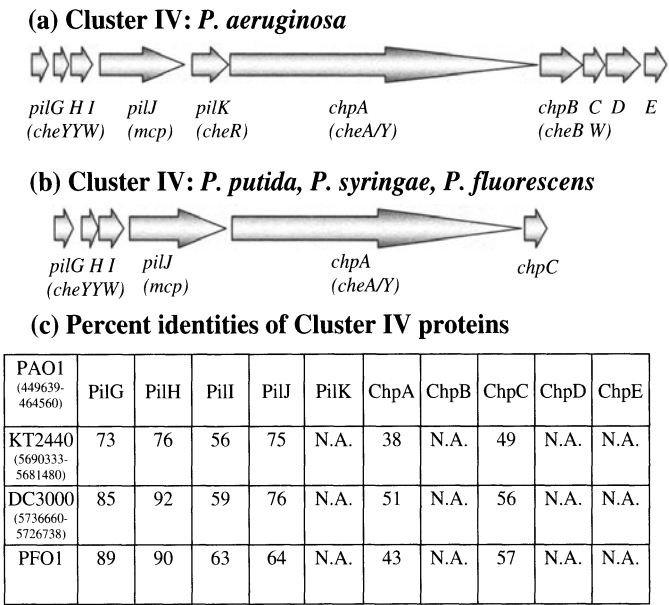


Figure 5. The gene organization of Cluster IV. (a) Gene order in *P. aeruginosa* PAO1. (b) Gene order in *P. putida* KT2440, *P. syringae* DC3000, and *P. fluorescens* PF01. Differences include the presence of *pilK*, *chpB*, *chpD*, and *chpE* only in *P. aeruginosa* PAO1. Homologous *che* genes are indicated in parentheses. (c) Percent amino acid identities of each of the Cluster IV proteins relative to the PAO1 deduced amino acid sequence. N.A.: not applicable (the genes are not present). The position of a cluster in a particular genome is given in parentheses under each strain name.

in *P. aeruginosa*^{54, 82}. The chemotaxis-like proteins encoded in Cluster IV appear to be part of a signal transduction system controlling twitching motility². PilG, PilI, and PilJ are required for normal pilus formation and twitching motility in *P. aeruginosa*, and although a PilH mutant retained these abilities, it had an abnormal motility pattern^{13, 14}. A PilJ mutant was also defective in directed twitching motility toward phosphatidylethanolamine, but since this strain was completely defective in pilus formation, the role (if any) played by the MCP-like PilJ protein in signal transduction remains unclear⁴⁰. PilK mutants were unimpaired in pilus formation and twitching motility even though *pilK* appears to be part of the Cluster IV operon¹⁵. It is possible that some other CheR homologue can compensate for the loss of PilK in *P. aeruginosa*¹⁶. ChpA, which is required for twitching motility, contains both CheA and CheY-like domains, and seven histidine phosphotransfer domains, making it one of the most complex signal transduction proteins known⁴⁵. Interestingly, mutants with transposon insertions in *pilGHIJK* and *chpA* demonstrated reduced virulence with *Drosophila* as a model host. This phenotype was not due to the

absence of twitching motility in these strains, because several other pilus and twitching motility mutants were fully capable of killing flies at wild-type rates¹². These results suggest that in addition to regulating pilus function, the Pil-Chp signal transduction system encoded in Cluster IV may also control the expression of an unidentified set of virulence factor genes.

3.5. Additional Chemotaxis Gene Clusters

Additional chemotaxis genes are present in Clusters VI, VII, VIII, in *P. putida*, *P. syringae*, and *P. fluorescens*, but not *P. aeruginosa* (Figure 6). Each cluster contains one or two recognizable chemotaxis-like genes. Cluster VI, which contain *cheR* and *cheB* homologues, is embedded in a cluster of ORFs with unknown function. Clusters VII and VIII each contain a *cheV*-like gene. The context of Clusters VI and VIII are identical in *P. putida* and *P. syringae*, but the context of Cluster VII is completely different in all three genomes. *P. syringae* has an additional chemotaxis gene cluster, designated Cluster IX. It contains a *cheW*-like gene and *mcp9*, which encodes a MCP. No information is currently available regarding the function or significance of any of the products of these clusters.

4. CHEMOTACTIC RESPONSES IN *PSEUDOMONAS*

4.1. How to Study Chemotaxis

Mutants with defects in chemoreceptors do not have obvious swimming defects visible under the microscope and more specific chemotaxis assays must be used for their characterization. Several types of assays have been developed to take advantage of the ability of bacteria to sense and swim up chemical gradients and accumulate near the source of an attractant. In the *soft agar swarm plate assay*, bacteria are inoculated at the center of a Petri plate containing growth medium solidified with a low concentration of agar (0.3%). Chemotaxis can be observed as a distinct ring of growth that gradually spreads to edge of the plate as cells swim up the concentration gradient created as they metabolize the compound. With this method, only metabolizable compounds can be tested as chemoattractants and the chemotactic response is dependent on the growth rate of the organism (i.e., mutants with growth defects may appear to be defective in chemotaxis). This assay is also useful for the enrichment and identification of chemotaxis mutants³.

Chemotaxis can also be measured with a *capillary assay*, in which a microcapillary tube containing a solution of attractant is placed into a suspension of motile bacteria in buffer¹. The attractant diffuses from the mouth of the

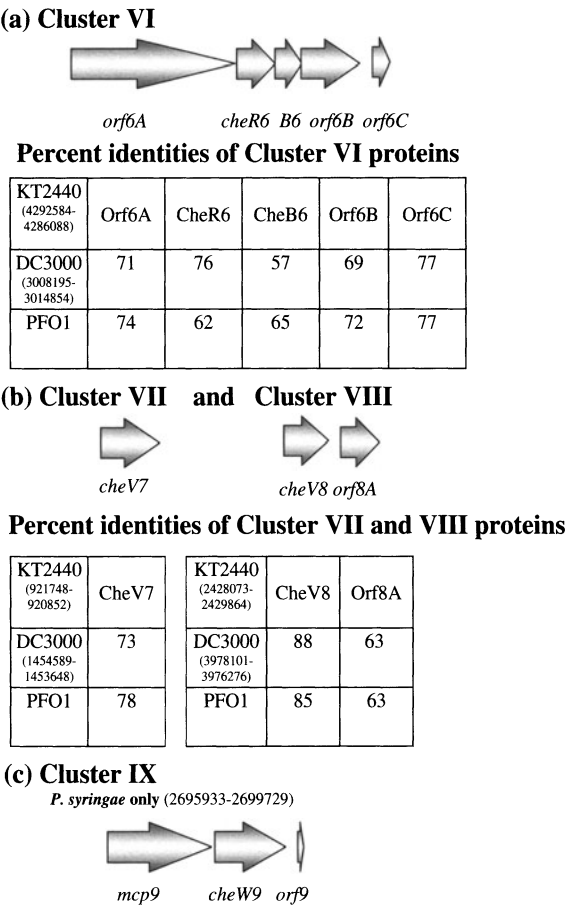


Figure 6. Gene organization of Clusters VI–IX. Clusters VI, VII, and VIII are present in *P. putida*, *P. syringae*, and *P. fluorescens*, but not *P. aeruginosa*. Cluster IX is unique to *P. syringae*. (a) Cluster VI gene organization and percent amino acid identities of each of the Cluster VI proteins relative to the *P. putida* KT2440 deduced amino acid sequences. (b) Gene organization of Clusters VII and VIII and percent amino acid identities of the Cluster VII and VIII proteins relative to the *P. putida* KT2440 deduced amino acid sequences. The position of a cluster in a particular genome is given in parentheses under each strain name. (c) Gene organization of the Cluster IX genes in *P. syringae*.

capillary into the buffer, forming a gradient, and chemotactic cells respond to the concentration gradient by swimming up the gradient and into the tube. The number of cells within the tube is determined, either by plate counts or direct microscopic counts⁴⁷ and compared with the number of cells that swam into a tube containing buffer only. A strong response is visible with a phase contrast microscope as a cloud of cells accumulates at the mouth of the capillary.

In this way, the capillary assay can also be used as a qualitative assessment of chemotaxis. We have been successful using the qualitative capillary assay to test chemotaxis to sparingly soluble compounds, such as toluene and naphthalene^{28, 56}.

Another qualitative chemotaxis assay is the *agarose plug assay*⁸⁴. This assay has proven useful for testing responses to volatile compounds such as toluene⁵⁶. Loss of the volatile compound is minimized due to the “closed” nature of the system. In this assay, a drop of melted agarose is mixed with a test attractant and placed on a microscope slide. A chamber is formed by placing a cover slip supported by two plastic strips over the agarose plug, and a suspension of motile cells is introduced into the chamber surrounding the solidified agarose plug. The attractant diffuses out into the cell suspension and a chemotactic response can be seen as a band of cells surrounding the agarose plug.

Finally, chemotaxis can be monitored in a *temporal assay*. Bacteria are too small to detect a chemical gradient directly; they actually measure changes in attractant concentration over time, and in a sense, have a primitive form of memory⁸. Because of this, the behavior of a population of bacteria can be monitored over time in response to the addition of a chemical attractant. Cells swimming in buffer change direction about 40 times per minute. When cells are exposed to an attractant, their swimming behavior changes and the frequency at which they change direction decreases. This attractant response results in “smooth swimming” cells that change direction much less frequently. After exposure to the attractant, cells “smooth swim” for a short period of time (typically 1–3 min). The temporal response can be quantified by either measuring the rate of change of direction of stimulated bacteria compared to that of cells swimming in buffer³¹, or the length of time it takes the population of cells to “adapt” or revert back to their previous swimming behavior⁶⁵. The response can be visualized microscopically and videotaped, and the behavior can be quantified either manually or with the assistance of a computer⁷¹.

4.2. Chemoattractants for *Pseudomonads*

P. aeruginosa and *P. putida* appear to be attracted to virtually all organic compounds that they are capable of utilizing as growth substrates²³ (Table 1). They are chemotactic to amino acids, sugars, organic acids and aromatic acids. *P. aeruginosa* strain PAO1 is also chemotactic to oligopeptides⁴¹. *P. aeruginosa* is chemotactic under anaerobic nitrate-reducing conditions as well as under aerobic conditions^{9, 23}. *P. putida* PRS2000 has served as a model for studies of chemotaxis to simple aromatic acids, like benzoate and 4-hydroxybenzoate that serve as good growth substrates for this strain^{23, 33, 34}. These responses are

Table 1. Chemoattractants for *P. aeruginosa*.^a

L-Alanine ⁴³	Gluconic acid ²³	Nonanoic acid ²³
γ-Aminobutyrate ⁷⁵	Glucosamine ²³	L-Ornithine ²³
α-Aminoisobutyrate ⁹	Glucose ^{9, 49}	Pelargonate ²³
γ-Aminovalerate ²³	Glutamate ^{48,49}	Phenylacetate ²³
Ammonium chloride ⁴⁸	Glutamine ⁴³	Phenylalanine ⁴³
α-Amylamine ²³	Glutarate ⁴⁸	Phenylpropionate ²³
L-Arginine ^{9, 48}	Glycine ⁴³	Phosphate ³⁸
L-Asparagine ⁴³	Glycerol ²³	L-Proline ⁴³
L-Aspartate ⁴⁸	Heptanoate ²³	Propionate ²³
Betaine ²³	L-Histidine ⁴³	Putrescine ⁷⁵
Benzoate ²³	4-Hydroxybenzoate ²³	Pyruvate ⁴⁹
Butanol ²³	DL-β-Hydroxybutyrate ²³	Quinate ²³
Butylamine ²³	4-Hydroxyphenyl acetate ²³	Sarcosine ²³
Butyrate ²³	4-Hydroxyphenyl propionate ²³	L-Serine ^{9, 49}
Cadaverine ⁷⁵	L-Isoleucine ⁴³	Spermine ²³
Caproate ²³	Isovalerate ²³	Succinate ^{9, 49}
Caprylate ²³	α-Ketoglutarate ⁴⁹	Threonine ⁴³
Citrate ⁴⁹	DL-Lactate ²³	trans-Aconitic acid ²³
DL-Citrulline ²³	L-Leucine ⁴⁸	DL-Trifluoroisoleucine ⁴⁸
Cysteine ⁴³	L-Lysine ⁴³	Trigonelline ²³
Ethanol ²³	Malate ⁴⁹	L-Tryptophan ⁴³
Fructose ²³	Mandelate ²³	L-Tyrosine ⁴³
Fucose ²³	Melibiose ²³	Valerate ²³
Fumarate ²³	Methionine ⁴³	L-Valine ⁴³
Galactosamine ²³	Mucate ²³	Vanillic acid ²³

^aReferences are indicated as superscript.

inducible and a dual function protein, PcaK, involved in both transport and chemotaxis of 4-hydroxybenzoate was identified^{19, 20, 32, 52}. The *pcaK* gene is located within a cluster of genes required for the degradation of benzoate and 4-hydroxybenzoate. PcaK is a member of the major facilitator superfamily of transport proteins. It is not clear at this time whether PcaK functions as the primary chemoreceptor for 4-hydroxybenzoate or if a MCP is also involved in the chemotactic response. The toluene-degrading strain *P. putida* F1 is attracted to toluene and a variety of aromatic hydrocarbons and related organic pollutants⁵⁶. The response is inducible and coordinately regulated with the toluene degradation genes, but the specific chemoreceptor has yet to be identified. Other *P. putida* strains are attracted to the polyaromatic hydrocarbon naphthalene²⁸.

A tomato root-tip colonizing strain of *P. fluorescens* (WCS365) is chemotactic to root exudates, some amino acids, malic acid, and citric acids¹⁷. Other strains of *P. fluorescens* have been shown to be attracted to sugars, amino acids and organic acids present in exudates of a plant pathogenic fungus⁶⁷. Only limited experimental work has been done on chemotaxis by *P. syringae*.

In one study, *P. syringae* VT10 was shown to be attracted to the growth substrates glucose, glycerol, and phenol, and also to maltose, a compound that it is unable to metabolize⁴⁶.

We know from studies of *P. aeruginosa*, *P. putida*, and *P. syringae*, that Pseudomonads are attracted to compounds that they are unable to metabolize^{28, 33, 34, 46, 48, 56}. Methylation of *P. aeruginosa* and *P. putida* proteins has been demonstrated in response to the addition of chemoattractants to cells and this was taken as early evidence that Pseudomonads process sensory information through MCPs in a manner analogous to *E. coli*^{10, 30}. Methylation of a specific *P. aeruginosa* MCP involved in chemotaxis to amino acids has been demonstrated more recently⁴³.

5. METHYL-ACCEPTING CHEMOTAXIS PROTEINS

5.1. MCPs in *E. coli*

Methyl-accepting chemotaxis proteins are receptors for chemical attractants, repellents and other sensory stimuli, such as oxygen^{72, 73}. *E. coli* has four MCPs for chemicals, which are constitutively expressed when cells are motile²². Two of them are present in cells at 10-fold higher levels than the other so-called “low abundance” MCPs. One (Tar) senses aspartate and repellents, and the second (Tsr) senses serine and repellents. A low abundance MCP (Trg) mediates sensory responses to galactose and ribose upon binding periplasmic binding proteins that are loaded with these sugars. Tap senses dipeptides. Each of the *E. coli* MCPs has separate binding sites for multiple attractants, repellents or periplasmic binding proteins, thus greatly increasing the total inventory of stimuli that are sensed by this relatively small number of receptor proteins. MCPs can bind and mediate chemotactic responses to compounds that *E. coli* does not metabolize. *E. coli* has, in addition, an aerotaxis receptor (Aer) that mediates an attractant response to oxygen^{5, 7}.

All *E. coli* MCPs with the exception of Aer have a common molecular architecture consisting of two transmembrane regions flanking a periplasmic ligand-binding domain in the N-terminal half of the protein followed by a conserved C-terminal cytoplasmic domain that is often referred to as the “highly conserved domain” (Figure 1). The highly conserved domain interacts with CheA and CheW to effect signal transduction in response to a conformational change that occurs when an MCP dimer binds an attractant or repellent. This domain also includes glutamate residues that are methylated or demethylated when cells adapt to a sensory stimulus. The aerotaxis MCP has a highly conserved domain at its C-terminus but it differs from the other MCPs in that it has a single transmembrane domain at the N-terminus, which serves to embed the protein in the cytoplasmic membrane. The N-terminal region of Aer

has a PAS domain that is associated with FAD. The FAD moiety has been proposed to mediate aerotaxis by interacting with a component of the electron transport systems⁶¹. PAS /PAC domains are typically involved in sensing redox potential, oxygen or light⁷⁶.

5.2. MCPs in Pseudomonads

Perhaps the best evidence that the Pseudomonads have highly developed sensory and response systems, is that they have large numbers of MCP-encoding genes present in their genome sequences. A summary of the numbers and types of MCPs found in the three completely sequenced species of *Pseudomonas* is given in Figure 7. *P. aeruginosa* PAO1 and *P. putida* KT2440 have 26 and 27 *mcp* genes, respectively, while *P. syringae* pv Tomato DC3000 encodes 49 MCPs, the largest number found in any completed microbial genome to date. An analysis of the incomplete *P. fluorescens* PFO-1 sequence indicates that it has

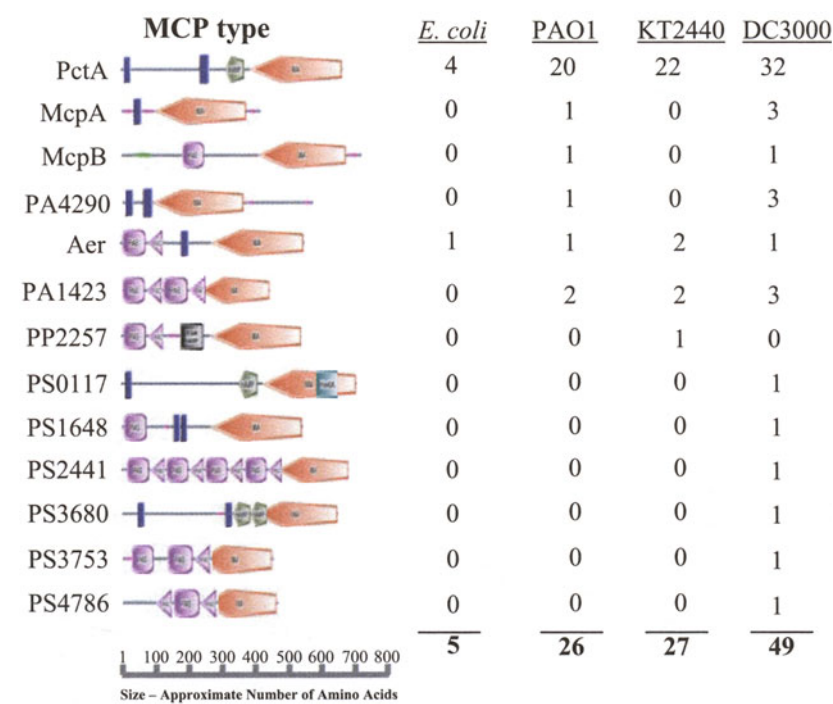


Figure 7. Number and types of MCPs encoded by *E.coli*, *P. aeruginosa* PAO1, *P. putida* KT2440, and *P. syringae* DC3000. Representative examples of each type from the *P. aeruginosa*, *P. putida*, or *P. syringae* genomes are indicated at the left of each structure. Diagrams were generated using the SMART Domain Search Program at <http://smart.embl-heidelberg.de>.

approximately 35 *mcp* genes. Most *Pseudomonas* MCPs have the traditional two-membrane spanning architecture and contain between 500 and 600 amino acids. However, some are missing obvious membrane spanning regions and are predicted to be soluble and to reside in the cytoplasm. Many of the predicted soluble MCPs have PAS/PAC domains and thus may possibly be involved in sensing some aspect of intracellular redox potential or energy status.

Only a few *Pseudomonas mcp* genes have been identified on plasmids. Three closely linked *P. aeruginosa mcp* genes, termed *pctA*, *pctB*, and *pctC* have been shown by the group of Ohtake to have overlapping specificities for amino acids. The PctA chemotaxis transducer was identified by the isolation of a serine chemotaxis mutant of *P. aeruginosa*⁴³. PctA was shown to be required for chemotaxis to glycine, serine, threonine, and valine, and a *pctA* mutant had very weak responses to most of the other L-amino acids. PctC, which is encoded upstream of *pctA*, detected histidine and proline, while PctB, whose gene is located downstream of *pctA*, was primarily responsible for detection of alanine, arginine, glutamate, lysine, methionine, tyrosine, and glutamine⁷⁵. Two MCPs required for inorganic phosphate (P_i) chemotaxis have been identified⁸³. CtpH and CtpL detected high (>5 mM) and low (0.01 mM) concentrations of P_i , respectively, and a double mutant did not respond to any level of P_i . All of these MCPs have a standard topology, with a positively charged N-terminus, two membrane spanning regions separated by a periplasmic domain, and conserved methylation sites in the cytoplasmic domain⁷⁵. One MCP, Aer, was shown to be required for aerotaxis in *P. putida*⁵¹. Aer from *P. putida* is only 27% identical in sequence to the *E. coli* Aer, but it has a PAS domain and a similar topology.

Several *mcp* genes have been identified in catabolic plasmids carried by various *Pseudomonas* strains. An inducible MCP, NahY, was identified in *P. putida* G7, a strain that grows on and is chemotactic to naphthalene^{28, 29}. The *nahY* gene is cotranscribed with the naphthalene degradation genes on the NAH7 catabolic plasmid. Inactivation of *nahY* resulted in a strain that was no longer chemotactic to naphthalene. NahY has topology typical of an MCP, but has very little amino acid sequence identity to other MCPs²⁹. Other MCP genes have been identified in degradation gene clusters. For example, the *alkN* gene, which encodes a MCP, is located within a cluster of genes for alkane degradation on the OCT plasmid in *P. putida* GPo1⁷⁸.

The functions of a few MCPs can be inferred from interspecies comparisons. For example, both *P. putida* and *P. syringae* have MCPs that are between 60% and 70% identical to *P. aeruginosa* PctB (PP1371 and PS1061), PctC (PP1371 and PS2480) and CtpH (PP2120 and PS2526). The three species encode very similar PilJ proteins, the MCP that is associated with pilus mediated twitching motility. The genes PA3708, PP1488 and PS1493 share between 70% and 75% amino acid identity and thus can be assumed to have very

similar, though at this point unknown, functions. In general, the deduced MCPs from the three *Pseudomonas* species do not have strikingly high (>60%) overall amino acid identities and this may reflect adaptations by the organisms to detect different sets of environmental signals in their different environmental niches.

It has been difficult to assign functions to specific MCPs in *Pseudomonads* by mutation analysis. Single knockout mutations in 18 of the *P. aeruginosa* PAO1 MCP genes did not result in a detectable chemotaxis phenotype after testing for responses to 68 organic substrates under aerobic and anaerobic nitrate-reducing conditions using soft agar swarm plates²³. Soft agar swarm plate screens to identify *P. putida* mutants defective in chemoattraction to 4-hydroxybenzoate have also not succeeded⁵⁷. There are a number of possible reasons for the difficulties encountered in making functional assignments to MCPs. The most obvious is that some have overlapping effector specificities. This is the case for PctA, PctB, and PctC and painstaking work was required to sort out which of these three MCPs has the strongest affinity for amino acid attractants that are, in fact, detected by all three MCPs⁷⁵. Some MCPs may be specific for inorganic compounds or for repellents. There is no good method available to rapidly screen for sensory responses to these classes of chemoeffectors. *P. aeruginosa* was shown to respond to the thiocyanate and isothiocyanate esters allyl isothiocyanate, ethyl thiocyanate, methyl isothiocyanate and methyl thiocyanate as repellents using a microscopic assay where cells were observed to swim away from the open mouths of capillaries containing repellents⁵⁵. MCPs responsible for the detection of these repellents have not yet been identified, however. A third possibility is that some MCPs are dedicated for use in a particular habitat or environmental situation such that they are expressed in a condition that differs from those used to screen for a chemotaxis phenotype. In a recent study, several *P. aeruginosa* *mcp* genes were found to be preferentially expressed in the stationary phase of growth⁶⁶. A final possibility is that MCPs may be used for a function other than chemotaxis. Such is probably the case for the *P. aeruginosa* McpA, McpB, WspA, and PilJ proteins. These are encoded by genes located in one of the “alternative” clusters of chemotaxis genes that do not appear to play a major role in flagella-based chemotaxis.

6. CONCLUSIONS

The application of bioinformatics to genome sequences has revealed many of the probable protein players that participate in *Pseudomonas* sensory behavior. However, the information gleaned from genome sequences also raises more questions than it answers. The main questions being: Why do *Pseudomonas* species encode such large numbers of chemotaxis proteins and

MCPs? And, what do they use them for? Detailed physiological analysis of mutants will be required to determine the functions of many of the MCPs. Determination of stimuli sensed by MCPs can inform us about what a particular *Pseudomonas* strain sees as being “important” about its environment. The multiple and diverse PAS-domain containing MCPs are especially intriguing, as many of them may be involved in communicating specific features of redox status. Approaches of whole genome gene expression (microarray analysis) are also expected to give clues to function because they will reveal *che* and *mcp* genes that are expressed under specialized sets of environmental conditions or in particular habitats. It will be interesting to determine whether some MCPs may be dedicated for use in guiding *Pseudomonas* species to enter into associations with plant, animal or insect hosts.

There is increasing evidence that the environmental sensing and signal transduction system of the type exemplified by the *E. coli* chemotaxis system has been adapted for uses other than chemotaxis. In the case of the *Pseudomonads*, the Cluster II and Cluster III *che* genes do not seem to have a strong involvement in chemotaxis, and instead appear to be involved in the attachment of cells to surfaces and aggregation of cells. Studies aimed at understanding how alternative sets of chemotaxis proteins function to direct cells to engage in behaviors like cell aggregation may reveal entire new layers of complexity in *Pseudomonas* sensory signal transduction.

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